Lawrence Berkeley National Laboratory

LBL Publications

Title

Scanning Electron Microscope Techniques in Biology

Permalink

https://escholarship.org/uc/item/7d39n74b

Author

Hayes, Thomas L

Publication Date

2023-09-06

eScholarship.org Powered by the California Digital Library
University of California

(7) Advanced Techniques in Biological Electron Microscopy

Scanning Electron Microscope Techniques in Biology

~~by~~ Thomas L. Hayes*

~~*Donner Laboratory, University of California~~

~~Berkeley, California, USA~~

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

A. Introduction

Fig 1

In contrast to the conventional electron microscope, the scanning electron microscope's utility does not generally lie in the area of ultra high resolution. Rather, it is the process of information transfer which is particularly aided by the scanning microscope.

This paper will attempt to describe some of the techniques of scanning electron microscopy (SEM) that have utilized this information transfer capability in biomedical research. The references are examples from the literature which happen to be familiar and significant to the author rather than a complete listing of the biological applications. For a more extensive bibliography of the SEM, the reader is referred to the excellent publications of WELLS (1967, 1968, 1969, 1970, 1971) or CARR (1971).

While the conventional electron microscope has extended our resolution very significantly, there are limitations on its ability to transfer chemical information and three dimensional images of complete biological units. The scanning electron microscope, although generally possessing lower resolving power than the conventional electron microscope, can help to fill some of these information gaps.

I. General Principles of Operation

The scanning electron microscope departs from the traditional spatial focusing method of image formation. Most images (light microscope, conventional electron microscope, telescope, eyeglasses, human eye) are formed by focusing radiation after it has left the specimen in such a way as to produce the necessary one-to-one correspondence between points on the specimen and points on the image. The scanning electron microscope utilizes instead a technique of painting the image through a time sequence of points,

a technique similar to that used in television imaging (OATLEY, et al., 1965).

The light microscope and the conventional electron microscope are related by the basic similarity of their imaging forming process. Many interesting points can illustrate the development of the conventional electron microscope as an extension of light microscope theory (COSSLETT, 1966). The SEM, however, is more related to the electron microprobe or to television imaging where all focusing takes place prior to interaction with the specimen and the image points are addressed in time.

The two basically different methods of imaging have been distinguished in the two instruments which produce pictures at a great distance. One long distance imaging instrument is a telescope and the other is television. It might be useful to also make this distinction in the imaging of small objects by referring to the light and conventional electron instruments as microscopes and to the SEM as microvision (HAYES, 1971_a).

The SEM might be described as consisting of two systems: the probing system into which the specimen is placed and the display system which forms the visual image (EVERHART and HAYES, 1972). In the probing system, electrons leave a hot filament or other source and are focused by means of electron lenses to a very fine point on the surface of the specimen. As the electrons interact with the material of the specimen at this point, they induce a variety of radiation such as characteristic X-rays, infrared, visible light, and streams of electrons.

These radiations leaving the surface of the specimen in a variety of directions can be counted and the measure of the number of photons or electrons of a specific type would reflect a particular quality of the specimen in terms of our knowledge of the production of that type of radiation by the primary bombarding electron beam. The direction in which a particular

radiation leaves is immaterial as long as that radiation is counted.

The measure of the amount of radiation leaving a point on the specimen at any instant can be used to modulate the brightness of the beam of the display cathode ray tube as it rests at the corresponding point on the image forming screen of this display tube. When the radiation from the point on the specimen is high, the point on the cathode ray tube would be bright.

If the probing beam is now moved to an adjacent spot on the specimen, information from that point can be used to modulate the adjoining point on the display cathode ray tube. In a similar fashion, a third and fourth point can be added in sequence until all points of the specimen have been covered in a regular array. The one-to-one correspondence necessary for the formation of an image occurs because of the single position of the probing beam corresponding to a single position of the display cathode ray tube beam at each instant in time.

In practice, the points follow one another with great rapidity so that the image of each point becomes an image of a line and the line, in turn, can move down the screen so rapidly that, to the eye, a complete image is formed. Such a process of the rapid sweep of an electron beam to produce a complete picture is familiar to us in television imaging.

If it is necessary to build up a better statistical sample of each point (EVERHART, 1970), a slower scan rate is selected and photographic film can be used to record each point and to integrate the entire picture over a longer period of time than can be integrated in a flicker-free fashion by the brain. Thus either through the direct visualization of a rapidly moving spot or by the collection over a long period of time of

many spots on photographic film, the end effect is an integrated picture of the specimen being studied.

In the familiar lens imaging system found, (e.g. light microscope, conventional EM), or in the mirror electron microscope (BOK et al., 1971), the information signal leaving the specimen is a vector quantity containing directional (localization) as well as amplitude and phase (information) components. In the scanning system, the information signal is a scaler quantity. All localization is a function of time and only the amplitude component is contained in the video signal. At each instant the scanning system needs only to measure the number of photons or electrons leaving the spot on the sample. It does not need to determine their direction.

The radiation leaving a single spot on the specimen at any instant is collected and then this signal is amplified and used to modulate the brightness of the cathode ray tube at that instant. There can be a complete separation in kind between the radiation used as the probing beam and the radiation that is collected and counted as the informational signal.

The two electron beams; one sweeping over the specimen, and the other sweeping over the face of the display cathode ray tube are in synchrony. They start at the top of their sweeps at the same instant, sweep through the same number of lines and end up at the bottom of their arrays at the same instant (HAYES, 1970a).

While the two beams sweep in synchrony over the same number of lines, the area of the array of lines (or raster) is not the same size on the specimen and on the face of the display cathode ray tube. In fact, the magnification of the scanning electron microscope is produced by having the display raster very much larger than the size of the synchronous raster sweeping the specimen. Magnification is defined as the ratio of the linear

size of the display raster to the size of the specimen raster. If, for example, the beam probing the specimen describes an array of lines one millimeter by one millimeter, and this information is related to points on the display cathode ray tube occupying a raster of ten centimeters by ten centimeters, the magnification of the system would be one hundred times. The useful range of magnification is dictated by the resolving power of the instrument (upper limit) and by the lens design and placement of the specimen (lower limit). The usual range of operation in scanning electron microscopy is of the order of ten times at the low end to fifty thousand times at the upper end. The range of magnification available to the scanning instrument puts it in a position between the light microscope and the conventional electron microscope, and in fact, the scanning instrument can act as a bridge between the other two.

II. A Comparison of Resolution

The resolution of a scanning electron microscope varies widely depending on the particular radiation being used as the information signal. The most commonly used signal is secondary electrons which produces the familiar three dimensional topographic image. Operating in this mode, currently available SEMs offer a resolution of about one hundred angstroms.

If transmitted electrons are utilized as the signal, resolutions below 5 Å have been achieved (CREWE et al., 1970; CREWE, 1971). If cathodoluminescence (visible light produced by electron bombardment) is used as the information signal, resolution may be no better than 1000 Å (PEASE and HAYES, 1966b, 1967)

Often the kind of information required in the image dictates the mode of operation and thus the available resolution. If, for example, the image must display the three dimensional relationship of a bulk specimen, the ultra-thin sections required for high resolution transmission scanning

are prohibited. Or again, if the biochemical information of cathodo-luminescence is needed, the somewhat higher resolution secondary electron image will not do. The type of information required in the image may take precedence over resolution and resolution as an end in itself becomes less important (HAYES, 1971b).

However, in any mode of operation the highest resolution is always sought (NIXON, 1968), and a consideration of some of the factors effecting resolution might be useful. The conventional electron microscope can achieve resolutions well below 5 Å. In spite of this remarkable achievement, it takes only a simple calculation to show that even when operated with this high degree of precision, the conventional electron microscope is utilizing only a small part of the resolution that is available based on the wave-length of the electrons alone.

The main factor which limits the resolving power of the conventional electron microscopes is not the wavelength-sensitive defraction, but rather spherical aberration of the electron lens (COSSLETT, 1966). In his elegant treatment of the factors effecting resolution in the conventional electron microscope, Zeitler has considered factors which range from the design parameters of the instrument itself to the physiological and psychological aspects of human vision (ZEITLER, 1969). It is pointed out that the conventional electron microscope today operates at a resolution limit in the 10 Å range and that the goal of research is pushing this resolution towards a 1 Å atomic resolving power.

The scanning electron microscope, when utilizing its most common mode of signal collection, can exhibit, at best, an order of magnitude less in resolving power, (100 Å). In a particular mode of operation, (scanning transmission electron microscopy), the SEM can, in fact, achieve

high resolution (CREWE, 1971) equal to that of the conventional electron microscope, but for the general operating conditions, the SEM ranks considerably lower in resolution than the conventional electron microscope.

The factors which control the resolution of the SEM in its most commonly used mode of secondary electron signal can be grouped into those factors associated with: (1) the limitations of a demagnified electron beam, (2) the limitations imposed by the interaction volume generated when the probing electron beam strikes the specimen, and (3) the limitations which are connected to design parameters dictated by the specimen conditions necessary for signal extraction (EVERHART, et al., 1959); (OATLEY, et al., 1965, ~~1968~~) (THORNTON, P. R., 1968); (BROERS, 1970).

Using general optical principles, Langmuir, in a classic paper, showed that the current density in a focused beam of electrons has an upper limit (LANGMUIR, 1937). If we express this maximum current density obtainable in terms of a minimum diameter of the probing beam of electrons even in a system free of aberrations, we would have a minimum diameter of the spot as expressed in equation number 1.

$$d_o^2 = \frac{4i}{0.6\,\pi J_c \alpha^2} \frac{kT}{eV} \qquad (1)$$

In this equation, d_o is the Gaussian probe diameter, J_c is the emission current density at the cathode, e is the electronic charge, eV is the electron energy, i is the probe current, α is the semiangle of convergence of the electron probe, k is Boltzmann's constant, T is the absolute temperature of the cathode.

In addition to this minimum diameter imposed by the basic electron optical considerations, there are also lens aberrations of the type found in the conventional electron microscope. These generally are listed under spherical aberration, chromatic aberration and astigmatism. There is also

a defraction limiting term involved in the determination of the final minimum diameter available. If we describe the diameter of a disk of confusion associated with each of these aberrations or limitations, we find that they are functions of the semiangle of convergence α, the wavelength of the electrons used λ, and the fractional energy spread of the beam $\frac{\Delta V}{V}$. These relationships can be expressed in the following equations (OATLEY, et al., 1965):

$$d_s = \tfrac{1}{2} C_s \alpha^3 \quad . \qquad \text{Eq. (2a)}$$

$$d_c = C_c \frac{\Delta V}{V} \alpha \quad . \qquad \text{Eq. (2b)}$$

$$d_f = \frac{1.22\lambda}{\alpha} \quad . \qquad \text{Eq. (2c)}$$

In equation 2, d_s, d_c, and d_f are the diameters of the least disk of confusion associated with spherical aberration, chromatic aberration and diffraction limits and C_s and C_c are the spherical aberration and chromatic aberration coefficients. Both C_s and C_c for magnetic objective lenses, have values comparable with their focal length. Astigmatism is a correctable aberration and will be treated in considerable detail in the section dealing with instrument operation.

In order to find the total probe diameter d, arising from the basic electron optical considerations expressed in equation 1 and from spherical aberration, chromatic aberration and diffraction as expressed in equation 2, the usual procedure (OATLEY, et al., 1965) is to add the respective diameters in quadrature:

$$d^2 = d_o^2 + d_s^2 + d_c^2 + d_f^2 \qquad \text{Eq. (3)}$$

$$d^2 = \frac{4i}{0.6\pi J_c \alpha^2} \frac{kT}{eV} + 1.5\lambda^2 \frac{1}{\alpha^2} + \frac{1}{4} C_s^2 \alpha^6 + C_c^2 \left(\frac{\Delta V}{V}\right)^2 \alpha^2 \quad .$$

Utilizing the above equation with certain reasonable assumptions, we can find an optimum value for α, and from this determine the resolving power for the conventional electron microscope as compared with the scanning electron microscope operated in the secondary electron signal mode (HAYES & PEASE, 1968). Such a calculation as carried out by Pease and others indicates that the resolution, $\frac{d}{2}$, of the conventional electron microscope lies below 10 Å, but for the scanning electron microscope, chromatic aberration is appreciable and a current of 10^{-12} amp. (about the minimum required for scanning microscopy) can be focused into a spot no smaller than 100 Å in diameter utilizing conventional sources (PEASE & NIXON, 1965). A part of the difference between the two instruments can be attributed to a higher spherical aberration coefficient and a lower operating voltage connected with the operation of a scanning electron microscope as compared to the conventional instrument. The higher spherical aberration coefficient results from the need to collect the low energy secondary electrons which emerge from the specimen surface. Such collection can only take place in field-free space and requires, therefore, that the focal length of the scanning electron microscope final lens is considerably longer than the lenses associated with the objective lens of a conventional electron microscope (about 1 centimeter in the SEM compared to 2 millimeters for a CEM). The larger spherical aberration and chromatic aberration coefficients limit the spot diameter (PEASE & NIXON, 1965).

The lower SEM accelerating voltage, V, is suggested by considering the interaction volume that occurs when the electron beam strikes the specimen. If the signal being utilized in the scanning electron microscope actually originates from all parts of this interaction volume, it is the diameter of this pear-shaped interaction zone that determines the resolution of the instrument rather than the diameter of the incident electron beams. In the

commonly used secondary electron signal mode of operation, the very soft secondary electrons are collected only from the volume very close to the surface and very near to the axis of the incident electron beam. This in spite of the fact that the incident beam can penetrate on the order of microns into the specimen, producing secondary electrons throughout this rather large volume. Due to their very low energy, the secondary electrons can only escape to be collected when they lie near the surface. This results in an effective collection diameter of about 100 Å for the secondary electron mode. If characteristic X-rays or visible light were being counted as the video information signal, the effective collection diameter would be considerably larger. Even in the case of secondary electrons, it is advantageous to limit the penetration of the electron beam by reducing the accelerating voltage.

The resolution of the SEM is determined by the probe diameter and by the factors related to the interaction between the probing electrons and the specimen. The relationship between resolution and contrast in terms of the important concept of signal to noise ratio has been presented in several of the papers by Everhart and collaborators (EVERHART, et al., 1959) (EVERHART, 1968) (EVERHART, 1970).

Utilizing what might be considered a standard scanning electron microscope (tungsten hairpin cathode, conventional oil diffusion pump vacuum system, and secondary electron information signal), Pease was able to obtain resolution comparable to the theoretical limit placed on electron probe size and current as determined by the aberrations of the lens, electron noise and contrast levels (PEASE & NIXON, 1965). The electron probe size achieved was approximately 50 Å and a resolution in secondary electron mode of 100 Å was demonstrated.

If we refer to equation 1, it is clear that advances in resolution could be achieved by utilizing a high intensity source in the electron gun. If the current density at the cathode can be increased, a smaller probe diameter with sufficient intensity to produce a picture in a reasonable time would be possible. A new high resolution design utilizing a lanthinum hexaboride cathode was presented by Broers in 1969. In addition to the high intensity gun, the effects of vibration, stray field and specimen contamination were reduced. Utilizing this specially designed instrument, Broers was able to demonstrate a minimum probe diameter of approximately 30 Å and a point-to-point resolution of approximately 50 Å in the secondary electron mode (BROERS, 1969).

The approach of Albert Crewe's group in Chicago has been directed towards high intensity guns coupled with an ultra-high vacuum system and the possibility of multipolar lenses; a combination that might be capable of resolving individual atoms (CREWE, 1966). The success of this program to date has been quite spectacular and, utilizing transmitted electrons as the information signal, Crewe has reported the visualization of individual uranium atoms (CREWE,[8] et al., 1970).

The SEM operated in the transmission scanning mode has been analyzed by applying the reciprocity theorem of optics, with the conclusion that this mode is capable of producing the same contrast mechanism effects as the conventional electron microscope (ZEITLER, 1971)[9]; (CREWE, 1971)[8]. This work pointed out some unique advantages of scanning transmission electron microscopy as compared to the conventional electron microscope; notably in the method of information presentation and the improvement of signal to noise ratio. Since the information is presented sequentially as an electronic signal, processing of the information is made considerably easier and because the

scanning transmission electron microscope can utilize all electrons interacting with the specimen rather than being restricted to elastically scattered electrons as is the usual case of the conventional electron microscope, we have an improvement possible in the signal to noise ratio.

Certain restrictions are also associated with scanning electron microscopy operated in the transmission mode. Since the signal being utilized consists of electrons transmitted through the specimen, the thickness of the specimen is reduced to the dimension familiar in conventional electron microscopy. Thus some of the depth information that can be valuable when studying a bulk specimen is not available in the scanning transmission mode. Also, the high intensity guns, in general, require considerably better vacuum conditions than the conventional hairpin filament. These conditions while improving specimen contamination, can also contribute to delays and inconvenience in specimen exchange and place restrictions on specimen outgassing.

The SEM as operated in its most general form (PEASE, 1971), currently has a usable resolution of approximately 1 order of magnitude less than a conventional electron microscope. There is available, however, the scanning transmission mode (CREWE, 1971); (KIMOTO, et al., 1969) which allows resolutions comparable to that of the conventional electron microscope to be obtained with certain restrictions as to the type of specimen and the operating conditions that must be used.

III. Comparison of Information Transfer

Fig 2

In comparing the information available with scanning electron microscopy, we will find that while the SEM has a lower resolving power, it can offer some additional modes of information transfer (HAYES and PEASE, 1969). Such information can be described as analytic and as subjective, or experiential.

1. Analytic Information

When the probing electron beam interacts with the material of the specimen, several types of radiation are produced and, in addition, alterations in the electrical properties of the specimen occur. Any of these radiations or alterations, if they occur essentially instantaneously, can be utilized to modulate the display cathode ray tube and thus paint out a picture of the specimen. Each of these pictures will contain information that is related to the way in which the secondary radiation has been produced. Among the radiations which have been utilized as a video signal are: visible light (PEASE & HAYES, 1966_b), (PEASE & HAYES, 1967), (MANGER & BESSIS, 1970), (KRINSLEY & HYDE, 1971), (REMOND, et al., 1970), (MUIR, et al., 1971), characteristic X-rays (RUSS, 1971), (TOUSIMIS, 1969), (JOHARI, 1971_b), (MUIR, et al., 1971), induced specimen current (EVERHART, et al., 1964), (EVERHART, 1966) and backscattered electrons in addition to the most commonly used signal, the low energy or secondary electron radiation.

The SEM, by utilizing these diverse radiations, can form images which identify chemical bonds, elemental composition, electrical properties, and topography of the specimens. Thus in terms of the types of interaction that can be recorded in the image, the SEM is somewhat more versatile than the conventional electron microscope.

There is also some advantage in the ability of the SEM to view bulk specimens. If geometric analysis is required in three dimensions, the rather difficult procedure of reconstruction from serial sections (STACKPOLE, et al., 1971) is necessary with the conventional electron microscope since each specimen observed in this instrument must be thin enough to transmit the electrons used to form the image.

Since many of the three dimensional geometries are of considerable importance in biology, the additional information available through the

ability of the SEM to sample depth and shape becomes important. This is particularly true in the areas of the nonmetric, nonprojective, enumerative geometries encompassed by the field of topology (RASHEVSKY, 1961).

2. Subjective or Experiential Information Transfer

The possibility of complementing objective, analytic information transfer with subjective, experiential aspects of image-observer interaction can be investigated using SEM techniques since several of these techniques mimic in their functional character the mode by which the individual experiences the world around him (HAYES, et al., 1969). Thus, the SEM can be utilized to extend our senses as well as to accumulate analytic data. Such an ability accounts for much of the attraction that scanning electron micrographs possess for scientists and laymen alike. In addition to being an attractive method of information presentation, such subjective contact can allow a more fundamental investigation of the usefulness of nonanalytic information transfer as a ~~useful~~ technique in the imaging of biological systems.

B. Specimen Preparation

The requirements of specimen preparation for scanning electron microscopy are no less rigorous and demanding than those of conventional electron microscopy or any other form of microscopic investigation (BOYDE & WOOD, 1969) (PFEFFERKORN, 1969) (PEASE, D., 1964) (BAKER, J., 1958). The investigator using the scanning electron microscope often finds the majority of his time spent in specimen preparation when compared to time spent in actual viewing with the microscope. Specimen preparation begins with the selection of appropriate tissue material and proceeds through the necessary fixation, dehydration and conductivity considerations.

I. Selection of Tissue

One of the most critical decisions to be made in any scanning electron

microscope study is to determine whether or not there is a reasonable chance that scanning electron microscopy can yield useful information about the subject. This decision depends not only on the material itself, but also on the kind of information that is required about the specimen. In many cases, it will be more appropriate to investigate the material using conventional electron microscopy, light microscopy, or electron microprobe rather than the scanning electron microscope. Limits of resolution, the fact that most of the techniques will reveal only the outermost surface of the specimens, and the severe environmental conditions that the specimen must experience in the column of the instrument, are factors that should be kept in mind. However, a large variety of biological materials of many types have been examined successfully. (CARR, 1971; BOYDE and WILLIAMS, 1968; BARBER and BOYDE, 1968; PEASE and HAYES, 1966a; PEASE and HAYES, 1968a; SMITH et al., 1971; THORNHILL et al., 1965; HAYES et al., 1966; GOLOMB and BAHR, 1971).

1. Natural Surfaces

Since the probing electron beam penetrates only a few microns into the specimen and in certain operational modes such as secondary electron, the signal emerges from an even thinner layer of the specimen, it is generally a surface that we are investigating in the scanning electron microscope. If a biological system contains information distributed on a surface that is in contact with either air or a solution, the preparation for scanning electron microscopy is relatively simple and does not require dissection or sectioning techniques. Such surfaces, however, must be considered quite carefully with respect to fixation and dehydration in order to prevent the introduction of unsuspected artifacts. At times the type of information that is required will remain invariant under certain deformations (HAYES, 1972), in particular, certain topological properties of shape will remain

Fig 3

invariant under many types of deformation. For most purposes, however, the surface integrity must be maintained without distortion and the proper handling of these surfaces through fixation and dehydration as outlined below is essential (BOYDE & WOOD, 1969).

The eye might be taken as an example of a biological system where considerable information is displayed on surfaces which are relatively accessible for scanning electron microscope viewing. The outer and inner surface of the cornea, the surface of the lens, the rather intricate filter system of the trabecular meshwork and even parts of the retinal complex can be prepared with little but simple gross dissection required. These surfaces of the eye are both cellular and noncellular and their study can yield information which can be correlated with conventional electron and light microscopy (SPENCER, et al., 1968). (SPENCER & HAYES, 1970). (BLUMKE, 1967). (KURABARA, 1970). (MATAS, et al., 1971).

Leaf surfaces (HESLOP-HARRISON & HESLOP-HARRISON, 1969), insect cuticle morphology (SOKOLOFF, et al., 1967). (HARTMAN & HAYES, 1971), and paleobiological samples (ERBEN, 1970), are other examples of specimens where information can be obtained directly with a minimum of dissection or sectioning.

Systems composed of individual cells offer surfaces which are readily accessible for scanning electron microscopy. Investigations of blood cells in both normal and pathological states (BESSIS & LESSIN, 1970). (KAYDEN & BESSIS, 1970). (CLARKE & SALISBURY, 1967). (SALISBURY & CLARKE, 1967). (WARFEL & ELBERG, 1970), tissue cultured cells (SHEIE & DAHLEN, 1969). (BOYDE, et al., 1968), and of protozoa (SMALL & MARSZALEK, 1969). (HORRIDGE & TAMM, 1969) are examples in this category.

2. Dissected material

Most biological material exists in three dimensional solid arrays which

do not offer the open surfaces discussed in the section above. For such material quite elaborate dissection techniques are necessary in order to reveal the parts of the specimen which are of interest. Lewis, in his work on neuronal tissues has developed several of these ultradissection and tissue clearing techniques (LEWIS, et al., 1969_a); (LEWIS, et al., 1969_b); (LEWIS, 1971); (HILLMAN and LEWIS, 1971). Extending the work of Thomas in conventional electron microscope preparations, (THOMAS, 1969), Lewis has also applied techniques of low-temperature ashing in the preparation of scanning electron microscope specimens. This procedure can help to clear the specimen of unwanted tissue components that would tend to obstruct the view in the scanning electron microscope and can be augmented by the introduction of nonvolatile components through the process of staining.

It has been suggested that a combination of dissecting and ripping of tissue following aldehyde-perfused and fixed tissue is a very suitable method for revealing internal surfaces (BOYDE and WOOD, 1969).

*

3. Sectioned Tissue

Fig 4

It is possible to utilize a standard microtome to prepare sections of biological material where the surfaces to be investigated lie deep within a solid organ or tissue. These sections can be viewed in the SEM after certain preparative techniques and with proper care in interpretation (ELIAS, 1971) can provide useful structural information. The preparative techniques of fixation, dehydration, and embedding often will not preserve ultrastructure in terms familiar to conventional electron microscopists but may retain some useful information at the cellular and very large polymer level. By investigating such sections utilizing the SEM, we can take advantage of the increased resolving power when compared with the light microscope and the ability of the scanning electron microscope to visualize the sides or vertical surface of these sections (MC DONALD, et al., 1967). Such sectioning technique has found application in the study of healing wounds (FORRESTER, et al., 1969_a) (FORRESTER, et al., ~~1970~~ 1969b), certain parts of the eye (SPENCER, et

*

The cytological techniques used to separate cell components can be utilized for the preparation of SEM material. Isolated stained chromosomes have been studied and their SEM morphology correlated with the light microscope image. (Sheldon and Traut, 1971)

al., 1968), central nervous system (MC DONALD & HAYES, 1967), (BOYDE & WOOD, 1969) and heart muscle (POH, et al., 1971).

The technique in general, consists of cutting paraffin sections (or utilizing slides previously prepared for light microscopy, POH, et al., 1971) after following the general procedures utilized in light microscope histological preparations. The sections are cleared in a solvent such as xylene, dried, metal coated and viewed in the SEM.

4. Living Specimens

A few organisms can retain sufficient water to survive the rather extreme environmental conditions (CROWE & COOPER, 1971) of vacuum and radiation encountered in the column of the SEM. Such specimens, while generally very much reduced in any physiological activity while being viewed, recover after removal from the column and present a unique type of specimen for study (PEASE, et al., 1966), (HUMPHREYS, et al., 1967), (HARTMAN & HAYES, 1971), (PEASE & HAYES, 1968). The preparative procedure for these specimens is minimal. In general, no fixation, dehydration or coating with conductive material is required. The elimination of preparative steps and the rather strict criteria of survival means that fidelity is probably quite good in these preparations.

5. Ion Etching

Ion etching, pioneered by Boyde and Stewart represents an additional technique for revealing underlying surfaces (BOYDE & STEWART, 1962), (STEWART & BOYDE, 1962), (BOYDE, 1967). In this procedure, a beam of ions such as argon is utilized to blast away the surface of the tissue in order to reveal the underlying material. It is necessary to try and distinguish between structures that have been revealed as a result of removal of the outer layers of material from those structures related to the ion etching process itself. Baker has considered some of the factors important to an interpretation of ion etched material, particularly in reference to the images of ion etched red blood cells (BAKER, R. F., 1970).

6. Freeze-Etching Techniques

Because of the obvious relationship in terms of surface investigation, the higher resolution freeze-etching technique utilizing conventional electron microscopy must always be considered as an alternative to the secondary electron mode scanning electron micrograph. The freeze-etching technique has proven to be of great usefulness in the investigation of surfaces where the qualities might be described as rough, but not too rough. That is, if the surface projections are relatively small (such as the particles that have been characterized on the surface of membranes), the freeze-etching technique can be of great value. However, if the surface becomes very convoluted, as for example the head of a beetle, it is difficult to utilize the freeze-etching replica to form an image of this entire very rough surface. Many aspects of freeze-etching technique have recently been reviewed by Koehler (KOEHLER, 1968).

The addition of complementary replicas techniques where both surfaces of a fracture can be compared (STEERE, 1971a) and the further enhancement of depth information by stereo-pair micrographs (STEERE, 1971b) has measurably improved our ability to interpret surface and shape characteristics by this technique. The depth of the replica is quite striking and the high resolution possible (30 Å) is bound to impress the scanning electron microscopist who has struggled to achieve a 100 Å resolution.

Depth and shape characteristics of the replicas can also be seen by scanning electron microscopy. It is possible to correlate the appearance of identical areas using SEM and CEM (MC ALEAR, et al., 1967). As will be discussed in further detail below, the freezing process is at times difficult to carry out without introducing artifacts of structure and the interpretation of replicated frozen surfaces can sometimes be difficult in the presence of the variety of particle shapes, holes and distortions produced by ice crystal

~~formation~~. In general, however, the freeze-etching technique offers an extension of resolution and an alternative method of imaging underlying surfaces that can be exposed in at least a semi-controlled fashion. Cryo-fracture without replication can also be a useful method for revealing specimen interiors (HAGGIS, 1970) (LIM, 1971). Frozen material may also be examined at low temperatures without dehydration (ECHLIN, 1971).

II. Fixation

1. Ultrastructure Fixatives

Although there are significant differences in terms of the embedding matrix and required resolvable detail between the SEM and conventional electron microscope preparations, the most often used fixatives for scanning electron microscopy are those adapted from conventional electron microscope techniques. For example, osmium tetroxide and OsO_4-$HgCl_2$ fixative (PARDUCZ, 1967) has been used very successfully to fix protozoa for SEM examination (HORRIDGE & TAMM, 1969) (SMALL & MARSZALEK, 1969). A formaldehyde-glutaraldehyde fixative of high osmolality first suggested for use in conventional electron microscopy by Karnovsky (KARNOVSKY, 1965) was utilized in a scanning electron microscopy study of the structure of avian lung (NOWELL, et al., 1970) and has found general application in our laboratory as a SEM fixative.

The fixation methods utilized in conventional electron microscopy are discussed thoroughly by Pease (PEASE, ~~DANIEL,~~ 1964) and certain of the specialized applications of fixation of biological samples for scanning electron microscope examination have been investigated (ARNOLD, et al., 1971) (ARENBERG, 1971) (BOYDE & WOOD, 1969)

2. Light Microscope Fixatives

Occasionally the fixative of choice is more related to standard light microscope fixation than to the specialized ultrastructural fixatives used

in conventional electron microscopy. Parducz's fixative (PARDUCZ, 1967) was originally developed for instantaneous fixation of the ciliates. Boyd has found that this fixative is very suitable when used to harden the free surfaces of soft tissues, isolated cells, or cultures (BOYD & WOOD, 1969).

The cross linking or precipitating properties of fixatives (BAKER, J. R., 1958) can be utilized to produce the desired degree of elasticity or brittleness in a tissue prior to dissection. Boyd has found that glutaraldehyde and formaldehyde make tissues tough, while Parducz's fixative makes them very hard and brittle (BOYDE & WOOD, 1969).

Finally, it may be useful to examine material which had originally been prepared for light microscopy (POH, et al., 1970). In this case, the preparative procedures are those associated with the standard light microscope preparation and as a result, the degree of fidelity is not comparable to the ultrastructural fixation and imbedding common to conventional electron microscopy. However, the advantages of having access to historical material prepared by standard light microscope pathology techniques may outweigh considerations of ideal fixation.

III. Dehydration and Drying

Dehydration, usually through a graded series of alcohols, is a familiar process in both light microscope and conventional electron microscope techniques. This dehydration is a necessary prerequisite to imbedding in the non-aqueous matrices utilized for the sections which are the standard specimens in these techniques. In scanning electron microscopy it is often necessary to carry out such dehydration for the purposes of freeze-drying from a non-polar solvent, or for substitution of a liquid which can be conveniently removed through critical-point drying.

In general, however, the specimens for the scanning electron microscope do not consist of sections, and therefore are not surrounded by any supporting

matrix. The need in scanning electron microscopy preparation is to remove the water completely rather than to substitute an embedding material for the water originally present. The techniques utilized for this removal of the water have generally been derived from the dehydration techniques utilized in conventional electron microscopy and have drawn heavily on the technology of the freeze-etch replica technique. A useful review of the preparative procedures utilized in the conventional electron microscope can be found in Pease's book (PEASE, D., 1964), and the techniques of freeze-etching have been covered in the recent review by Koehler (KOEHLER, 1968).

1. Freeze-drying

Freeze-drying has probably been the most often used technique to remove water from scanning electron microscope preparations with a minimum of artifactural change. Although freeze-drying has sometimes been referred to as an "artifact-free" method, it is as prone to difficulty in this direction as other ultrastructure preparative techniques (KOEHLER, 1968) (BOYD & WOOD, 1969). The artifacts of preparation may arise during the freezing process or during the sublimation of the ice under the low temperature, high vacuum conditions of this technique.

After considerable experimentation, Boyd and Wood have concluded that the tissue surface is least distorted following freeze-drying from a non-polar solvent, e.g. Amyl acetate can be substituted for the tissue water. Freeze-drying from water is a relatively slow process requiring days or even weeks of vacuum evaporation at low temperatures (-70°C). By substituting the non-polar solvent, the freeze-drying time can be shortened to a matter of under one hour (BOYD & WOOD, 1969). A second advantage is that the non-polar materials freeze more in the form of an amorphous glass than

of a typical ice crystal. Since ice-crystal formation is one of the most serious problems associated with freeze-drying, substitution of organic liquids for the water is a definite advantage. At the same time the possible chemical alterations produced by changing the chemical environment from water to a non-polar solvent must be considered a drawback.

Several commercial freeze-drying units are available. It is advantageous that the unit be equipped with an electronically cooled stage where the temperature of the specimen can be monitored continuously and a specimen chamber that is free of contamination produced by outgassing of chamber components or back-streaming of oil vapor from the mechanical pump. Since the specimen temperature is often maintained in the -70° to -80°C range it is also useful to have a cold trap at liquid nitrogen temperatures to assist in reducing the contamination on the surface of the material during freeze-drying.

Upon completion of the freeze-drying (approximately 3 days for specimens of 1-3 mm. in thickness, BOYD and WOOD, 1969), the specimen is warmed to slightly above room temperature before air is readmitted to the vacuum chamber. Such warming is necessary to prevent condensation which might re-hydrate the specimen.

The specimen can now be stabilized by coating with a thin layer of carbon, which will help to maintain the dried state for specimens which are hydroscopic. Most specimens also will be coated with a thin layer of conducting heavy metal by evaporation techniques (see below). Boyd has developed a vacuum station in which both freeze-drying and the evaporative steps associated with the carbon and heavy-metal layers can be carried out in a single chamber during one vacuum cycle. Such a procedure eliminates the danger of rehydration, but the very long times associated with freeze-drying means that the evaporation unit will be tied up for long periods of time and not

available for other uses.

2. Critical Point Drying

A second method for removing the water from the specimen without the damaging effects of surface tension is the critical-point technique developed by Anderson (ANDERSON, 1951). In a two-phase system such as the liquid water in equilibrium with the gaseous water vapor over the specimen, there is a point in terms of the environmental temperature and pressure applied to these two phases where the liquid phase will become indistinguishable from the vapor or gas phase. At this point the liquid will be converted to the gas which can then be removed from the system without the surface tension effects associated with air drying. The values of temperature and pressure, where this transition occurs, is called the "critical point."

Water is not a practical material for the critical-point process because of its extremely high pressure and temperature values; therefore, the water of the specimen is replaced; first, by an intermediate liquid and then by the transitional liquid having the critical point within the range of design characteristic of a laboratory apparatus. Anderson utilized ethyl alcohol as the dehydrating agent, amyl acetate as the intermediate liquid, and carbon dioxide as the transitional liquid. Boyd has utilized this method in the construction of a critical-point apparatus for scanning electron microscopy. A detailed description of the apparatus and its use is contained in his article describing preparative techniques (BOYD and WOOD, 1969). He found generally satisfactory results but noted a 10% bulk shrinkage artifact with this method, as measured in certain tissue samples.

The lower critical point associated with the fluorocarbons, "freons," has been utilized by Cohen in the construction of a critical-point apparatus using readily available commercial components and capable of rapid and efficient operation (COHEN et al., 1968).

At least three commercial models of critical-point apparatus which operate with CO_2, freon, or both, are now on the market.

A comparison of freeze-drying and critical-point drying as preparative techniques for scanning electron microscopy is demonstrated in two papers dealing with the structure of protozoa. The freeze-drying technique was utilized in one (SMALL and MARZALEK, 1969), while the other utilized the critical-point technique for drying similar osmium fixed protozoan specimens (Horridge and Tamm, 1969). The two methods can be used together in order to identify freezing and chemically induced artifacts.

3. Air Drying

The surface tension effects associated with drying in air in general distort and obscure the details of the specimen. Occasionally however, this surface tension flattening can be an advantage (e.g. in the disclosure of internal granules of a cell which would not be revealed if the spherical form of the cell were maintained through freeze drying or critical point drying, MC DONALD and HAYES, 1969). Cell junctions may be emphasized by air drying (SPENCER and HAYES, 1970), and the A, I, and Z bands in striated muscle fibers can also be accentuated by this technique. Boyd and co-workers have demonstrated that sub-surface features such as nuclei and mitochondria can also be revealed (BOYDE et al., 1968; BOYDE et al., 1969). Air drying is useful when certain kinds of information are to be extracted from the specimen preparation (e.g. comparative form, ROTH, 1971) but in general it cannot be substituted for the more faithful methods of freeze drying and critical-point drying.

IV. Improving Conductivity

Since it is generally desirable to maintain the electrical potential of the specimen surface at ground level, the specimens for scanning electron microscopy are usually coated with a thin layer of conducting material by vacuum evaporation techniques prior to viewing in the instrument. Most

Fig 6

biological specimens in the dehydrated state conduct electricity rather poorly and this layer prevents the build-up of beam induced charge and the resulting artifacts. Living material, where the presence of the hydrated system seems to prevent an accumulation of charge (PEASE, et al., 1966) is an exception, usually requiring no metal coating. The interpretation of the bright areas and image distortions produced by charging has been discussed by several authors (BOYD & WOOD, 1969), (PAWLEY, 1972).

1. Metal Evaporation

~~a) Choice of metal~~

Most frequently the conducting layer consists of a layer of carbon, which provides for a well covered surface, followed by an outer layer of heavy metal, usually gold, that has been evaporated on the sample in order to improve the secondary electron coefficient and to limit somewhat the interaction volume. In order to achieve uniform coverage it is essential to rotate the specimen quite rapidly. The carbon layer (not more than 200 Å thick) is tough and stabilizes the specimen while providing a good substrate to which the gold layer is firmly adherent. Carbon is easy to evaporate, causes little specimen heating, and is easily scattered by the residual air molecules to form a continuous layer over the specimen. Gold is very easy to evaporate from a tungsten filament, has a reasonably small granularity by SEM standards and has a high secondary electron emission coefficient (BOYD & WOOD, 1969).

At very high scanning electron microscope magnifications the granulation of the gold film is sometimes observable and may become a problem. In order to reduce the granularity the metal coatings utilized in high resolution conventional electron microscopy can be used for scanning electron microscope preparations. Very small granularity and relative ease of evaporation from tungsten is possible with a platinum-palladium alloy or with chromium.

Specimen damage sustained during the metal evaporation procedure due to heating is probably quite small. Koehler has discussed the temperature rise associated with radiant heating and the heat associated with phase changes experienced by the condensing metal on the specimen surface. For most evaporation procedures it would seem that the temperature increment at most is one or two degrees Centigrade which is far below that necessary to produce volatilization of the components of the dried tissue sample (KOEHLER, 1968).

2. Conducting Sprays and Solutions

For certain studies it is possible to spray an antistatic agent onto the specimen as an alternative to metal evaporation (SIKORSKY et al. 1968). The antistatic agent, (e.g. Duron) can be applied very conveniently and without exposing the sample to the vacuum of an evaporator. It tends, however, to cover small surface detail and therefore is most useful at the lower magnifications.

A liquid such as a fatty acid mixture can also be applied to the specimen surface in order to improve conduction under electron bombardment. Again, residual material can obscure fine detail and introduce unwanted artifact. In one of the early studies of mammalian tissue, soaking of tissue specimens in solutions of metal salts was tried with only moderate success. (JAQUES, et al., 1965).

C. Viewing Techniques

The actual operation of a scanning electron microscope for biological investigation can be carried out by the investigator himself or through the use of a specialized technical operator. The wear and tear on an instrument is, of course, increased by having a multitude of different operators, some of whom have relatively little experience in the operation of intricate electronic apparatus. However, if the operator is utilized there is always the difficulty of the biologist working "over his shoulder." The technical operator is more proficient in the operation of the instrument but may lack

the biological background in the specialty of the investigator. This background often seems important in the interpretation of images and in the selection of the field, magnification, viewing aspect, etc.

Often the scanning electron microscope represents a joint investment of several departments or divisions which may include the physical sciences, engineering, biology, and the medical sciences. Instrument design, which in the early days of the commercial instrument was directed rather exclusively toward the engineering and physical requirements, has been extended to include instrumentation designed more directly for the needs of the biological science investigator. Several companies have instruments which are directed toward the operation by the biological investigator himself, and if price considerations can be improved, it would be hoped that small scanning electron microscopes ~~can~~ will be available, not only within the biological departments but even, perhaps, in the individual laboratories of the biological investigators.

As the operator faces the scanning electron microscope, it is apparent that there are many variable operating parameters that must be considered in the production of the image. In order to feel comfortable with all of the variables involved, it is necessary to sit down and actually operate the instrument. During this learning period some of the following suggestions may prove useful.

I. Standard Specimens

If we are to make reasonable progress in our attempt to integrate the various operating parameters it is very useful to have a specimen which is stable and contains morphological detail that can be used to test the operation of the instrument.

Standard specimens which are biological in nature have the advantage in that their density, surface texture, and chemical composition is more directly

related to those samples which will probably be under investigation. Manning has shown that tobacco mosaic virus paracrystals and serum lipoprotein macromolecules might be used as standard specimens (MANNING, et al., 1968).

In general, however, non-living material offers a much greater specimen stability, and can be used for repeat testing over long periods of time. Mineral samples can be prepared with surface structures that test the maximum resolving power of the instrument, and with a judicious choice of density and elemental composition we can still approximate the composition of biological material.

We have found that a sample of precious opal, that has been etched with hydrofluoric acid and then coated with a conducting layer by evaporative techniques (JONES, 1969) is a very satisfactory standard specimen. Such a specimen remains stable over many months and contains structure at two size levels: one corresponding to the moderate range of magnifications of a scanning electron microscope and a fine structure that is near the resolution limit of our present instruments. Opal has been investigated by light microscope and conventional electron microscope techniques (JONES, et al., 1964; DARRAGH, et al., 1966; SANDERS, 1964) and thus has the additional advantage of presenting a well-established structure that has been identified by several imaging methods.

II. Signal Monitor

If we are to make the necessary quantitative appraisal of the performance of the scanning electron microscope it is essential that we have a method for monitoring the information signals (PAWLEY, et al., 1969). Most commercial instruments have this capability to a more or less satisfactory degree. The signal monitor acts as an exposure meter for the photographic recording of the image and the signal displayed should represent as nearly as possible the

amount of light at each point on the display cathode ray tube. If the monitor displays this signal, both the brightness and the contrast levels can be read directly, and no subjective estimates or dependence on prior experience is required for efficient photographic recording. Completely automatic photographic systems are also now available.

By observing a single line of the scan (utilizing a vertical defeat circuit) and sharpening the peaks of the display a best focus position can be determined. Such focusing is particularly useful at very high magnifications where the required low specimen current for maximum resolution often means that the image will be quite reduced in intensity.

The monitor can also help in the analysis of signal distortions produced by stray fields, electronic failure, or mechanical vibration. Identification of the difficulty is often aided by an ability to determine both the frequency and the amplitude of the imposed distortions.

III. Accelerating Voltage

The operator generally has a considerable range of accelerating potentials from which to select, accelerating potentials from 1 kV to 50 kV being most often utilized. It has been suggested that for the observation of biological material accelerating voltages no higher than 10 kV be used. Adequate resolution, at least for certain microscopes, may only be obtained at a higher accelerating potential and some experimentation is usually in order. It is probably best for the operator to try several potentials, keeping in mind that such things as increased interaction volume and the charging effects produced require careful interpretation. If this is kept in mind, it seems that for certain samples the increased signal brightness produced by slight charging effects at higher voltages might actually aid in the visualization of some detail.

IV. Specimen Current

The specimen current selected by the operator should be as low as

possible while still enabling the formation of a relatively noise-free image. The ability to measure specimen current is quite useful and the micro-microammeter usually used for this purpose is often consulted. The probe diameter is controlled in part by the specimen current (see Equation 1), and for the usual high resolution work currents in the range of 1 or 2 x 10^{-11} A are desirable. For survey work at somewhat lower magnifications specimen currents in the 10^{-10}A range are often useful, since they provide a considerable increase in the signal and therefore improve noise considerations.

Fig 7

For operational modes other than secondary electron collection it may be necessary to increase the specimen current even further. Cathodoluminescence and characteristic x-ray images can be produced in general only with a considerable increase in the specimen current. Resolution in these modes is generally determined by considerations other than probe diameter, and specimen currents as high as 10^{-8} or 10^{-7} amps are commonly utilized.

V. Contrast; Photo-multiplier

In operating a scanning microscope the contrast of the image is often controlled by regulating the voltage of the photomultiplier tube. Contrast within the range of recording film can be set subjectively, utilizing the display cathode ray tube, or can be determined by measuring the peak height of the signal monitor display. If a process of re-photographing is to be used, the possibility of increasing contrast through successive photographic reproduction should be considered. The selection of a contrast level somewhat below that which might appear optimum on the display cathode ray tube is suggested. Extreme contrast in the image is often a result of charging and is best corrected by improving the conduction of the sample (see B, IV above).

VI. Scan Rate

Frame speeds ranging from hundreds of seconds to TV rates (1/30th of a second or faster) can be utilized in scanning electron microscopy.

The slower scan rates are used when it is necessary to extract statistically significant information for each of the picture elements utilized in a photographic recording of the image. Typically a frame speed (or exposure time) of about 40 seconds has been used. However, a 40-second frame cannot be integrated by the brain and for direct viewing and for the observation of dynamic techniques the much faster sweep speeds are called for.

Television scan rates (KIMOTO, et al., 1969) are very useful for focusing and for observing astigmatism and movement of the specimen. Survey work, that precedes a more detailed high magnification study, also benefits from flicker-free display. TV scan rate helps to correlate adjustment of the focus control with the instantaneous image; eliminating confusion with the long-persistance image used for visual observation of lower scan rates.

In order to assess specimen movement in a smooth and accurate fashion it is necessary that the frame speed approach standard TV rates and the use of a micromanipulator in the stage of a scanning electron microscope can only be accomplished if the images can display movement. For this type of microdissection it is essential to be able to observe the specimen and the microdissection needles in the TV scan rate mode.

VII. Astigmatism Correction

The process of astigmatism correction, possibly more than any other operator function, will determine the resolution of the final image.

The most effective way to deal with astigmatism is to prevent its occurrence. Astigmatism often is the result of contamination in the column of the microscope, in particular, contamination of the final aperture. The best resolution is obtained when the column is absolutely clean and a minimum astigmatism correction need be applied. However, in day to day operation a small astigmatism correction is generally necessary.

Any astigmatism correction contains two parameters: one associated with the magnitude of the correction and the other associated with the direction in which this correctional field will be applied. The fact that two variables are concerned in astigmatism correction makes it somewhat more difficult to manipulate than a single variable adjustment such as focus. The purpose of the astigmatism correction is to introduce into the electron optical system an artificial astigmatism which will be equal in magnitude but opposite in direction to the astigmatism that is inherent in the microscope at the moment.

Fig 8

The astigmatic system will focus one direction in the specimen plane at a different focal plane than the orthogonal specimen direction. By determining the direction of low and high focus the azimuthal characteristic of the astigmatism of the microscope column can be determined. It is sometimes helpful in this respect to utilize a plastic overlay that can be rotated and set to record the direction of the astigmatism. The next step is to locate (with the azimuthal control of the stigmator) the proper setting which will produce an astigmatism that is opposite in direction to that found in the instrument. To do this the magnitude control of the stigmator is increased until the introduced astigmatism is considerably larger than that present in the instrument so that the direction can be calibrated independently of any astigmatism that is present in the instrument.

Once the direction of astigmatism correction has been determined it is next necessary to introduce the proper magnitude that will exactly cancel the inherent astigmatism. Some idea of the magnitude of astigmatism present can be gained by observing the difference in focus steps between the low focus and high focus directions. Most often the magnitude of the astigmatism correction is set while observing the image at rapid scan rate, increasing the magnitude of the astigmator from zero until the best image is obtained. If

~~the magnitude of the astigmator from zero until the best image is obtained.~~ If the magnitude of the introduced astigmatism is too low, the astigmatism direction will be that of the original determination; if it is too high the direction will be that of the introduced correctional astigmatic field.

VIII. Final Aperture Size

The selection of a final aperture is dictated by the magnification, (resolution) and by the depth of focus that is required. For very high resolution work, a large aperture is appropriate since high magnification observations are limited in terms of height variations. At low magnifications a large depth of focus is often a prime requirement and for this purpose we should use a smaller final aperture (large objective aperture is possibly 200 microns and a small aperture might be 50 microns, BOYD and WOOD, 1969). As noted above, the cleanliness and centering of the final aperture is essential if astigmatism of the system is to be low. It is a considerable advantage of instrument design if the final aperture diameter can be selected and positioned conveniently.

IX. Viewing Aspect

The aspect of the specimen shown in the secondary electron image of a scanning electron microscope is a projection of the specimen on a plane perpendicular to the probing beam. If the specimen has structure that is intricate in the sense that certain parts are hidden in this projection, it will be necessary to rotate and tilt the specimen in order to see all parts of it.

A goniometer stage of high mechanical stability and good range in the three translational directions as well as rotation and tilt is an important component of the microscope. In addition to increasing the number of surfaces which can be visualized, an accurate tilting stage allows the production

of stereo-pairs (BOYDE, 1970). Special techniques for manipulating the specimen might also be considered (BYWATER & BUCKLEY, 1970).

If the stage is tilted through an angle of 8 to 10 degrees between successive photographs, the two photomicrographs will represent information that can be compared to the two viewing aspects of human binocular vision. Such stereo-pairs allow analytic processing that cannot be achieved through any single photograph (WELLS, 1960); (BOYDE, 1970). The stereo-pair images can be recombined by several techniques, analytic and subjective (see also section E, II below) and represent a very large increase in both analytic and experiential contact with the specimen.

X. Micromanipulation

Pawley has designed and constructed a micromanipulator that can be used within the column of a SEM (PAWLEY & HAYES, 1971); (PAWLEY, 1972_a); (PAWLEY, 1972_b). This micromanipulator utilizes piezo-electric crystals to move two small needles in an independent fashion over very small distances. These needles can be positioned to within 0.1 microns and their response time is very short allowing for joy stick control and good coordination with the hands and eye of the operator. The micromanipulator has proven valuable where dissection or tearing of the specimen is required and has also been used to introduce a microprobe that can be utilized in the study of the charging artifact (PAWLEY, 1972_c)

Fig 9

D. Signal Processing

I. Differentiation.

In order to compress the signal range and to emphasize characteristics as a function of the rate of change of the signal rather than the signal level itself, several applications of electronic differentiation have been utilized (CREWE et al., 1969); (HEINREICH et al., 1970). Differentiation can be utilized as a mathematical tool related to the "lighting direction" as seen by the observer. If the derivative function of an object is considered, we find that the signal itself may approximate a square pulse, but that the derivative

of the signal would consist of two peaks, one positive and one negative. The combination of a positive and negative derivative signal when utilized to modulate the display cathode ray tube, results in "lighting" the object from one side. Thus, where the derivative is positive, the sample would appear bright, while on the opposite side of the sample, there would be a dark area corresponding to a shadow. A mixture of normal signal and differential signal can sometimes be useful in improving the transfer of shape and depth information aiding in identification.

II. Deflection Modulation

Everhart, Wittry, and others have developed signal deflection techniques which aid in analysis and can generate striking three-dimensional displays (EVERHART, 1966); (WITTRY & VAN COUVERING, 1967). The original signal may have no depth codes (i.e., the specimen-induced current) but may be more effective if displayed or transformed into a spatial mode. Deflection modulation utilizes the information signal to deflect the beam of the display cathode ray tube rather than to change its intensity as is the usual case in intensity modulated images. This process converts the signal strength into apparent height of a solid three dimensional object. Relationships between signal intensities can then be measured in terms of this deflection and at times additional insights into the signal image characteristics are possible.

Defelection modulation has also been utilized in connection with the secondary electron signal in order to emphasize surface texture. It should be noted, however, that in contrast to secondary electron depth coding which is basically a function of the angle between the beam and the specimen at each point, the deflection modulation "height" is an arbitrary function and is completely at the disposal of the operator. To say, therefore, that deflection modulation increases the resolution of height is misleading.

Resolution in height (Z direction) of the specimen will not be effected by this method of presentation (modulation) of the image. It is quite true that deflection modulation may make certain height relationships over the surface of the sample more apparent and more easily correlated in the mind of the observer, but the basic distances that can be separated in terms of Z resolution are not effected by the generation of an artificial texture through deflection modulation.

III. Color Modulation

In the previous section we have considered utilizing the information signal to deflect the beam of the display cathode ray tube. It is also possible to utilize signal strength to determine the color of each point of the image. In such a color modulation system (PAWLEY, et al., 1969) a given signal intensity is converted to a particular color. In color modulation, the assignment of a particular hue or color to a particular signal intensity is at the disposal of the operator, but once this assignment is made it is the signal itself as it is generated at the specimen surface that will determine the color pattern of the final picture (HAYES, et al., 1969).

Color modulation can be useful in depicting iso-intensity areas of the image and can also help to accentuate gray level steps that are too small to be recognized without such color coding. By contrasting color between two adjacent gray levels, patterns of signal distribution can be recognized more easily (EVERHART and HAYES, 1972).

Color has also been utilized to code two or more separate information signals collected simultaneously for image display. This process, generally referred to as color coding to distinguish it from the color modulation described above, has been utilized for example, to combine the cathodoluminescent and secondary electron images in a single picture.

IV. Computer Processing

Certain of the image processing techniques found to be useful in light and conventional electron microscopy (FRANK, 1972) (BURKE et al., 1971) and several aspects of transfer theory (BUDINGER, 1971) are applicable to SEM image evaluation. Because the information from the SEM is presented as an electronic, time sequence signal, direct processing by computer is a possibility. In addition to processing the image, the computer may be utilized to control the microscope and generate the raster for the scanning beam. McDonald has reported a computer SEM system utilizing an IBM 1800 computer which processes the video signal and also generates the raster (MAC DONALD, N. C., 1968). Pattern recognition programs may be utilized to analyze information from the micrographs (LIPKIN AND ROSENFELD, 1970) and stereometric analysis has also been carried out using computational methods. (DORFLER and RUSS, 1970) (BRAGGINS, et al., 1971).

E. Recording Techniques

Recording techniques serve to provide a permanent picture that can be reviewed and analyzed and also serve to integrate an image which may be too slow for physiological response time. For specimens where no motion takes place, recording media are generally films or Polaroid positives and for the recording of dynamic ~~technique~~ phenomena, video tape is the commonly used recording media.

I. Photographic Integration

1. Polaroid Film

Since many of the photographs taken with the SEM are for the purpose of allowing the observer to see an integrated high resolution image, Polaroid positive roll film (Type 42) is often chosen. Since intensity of the display cathode ray tube is not a problem, very fast film is not required. When an

image needs to be processed for publication, display, teaching purposes, etc., the positive Polaroid print can be quite satisfactorily re-photographed and the negative produced in this process can be stored for future use. The fraction of photographs, in our experience, that need be rephotographed is small, and the use of positive film is somewhat more economical than the production of negatives directly from each SEM recording through the use of Polaroid PN (positive-negative) film. However, if re-photographing is not convenient or if the percentage of the images where negatives are required is quite high, the PN film can be the preferred method.

Polaroid Polacolor film has been utilized to record color modulation and color coding images (PAWLEY, et al., 1969) utilizing the visible light spectrum from a standard CRT display tube. Probably of more general use, in the sense of color conversion, are photographic processes which allow the color conversion to take place away from the microscope itself. This separation provides more latitude in the production of the color of the final color print and also frees the microscope for other uses during the darkroom color conversion.

2. 35 Millimeter Standard Roll Film

The savings in cost of film are considerable if a 35 mm standard roll film is used instead of Polaroid. The obvious loss is the immediate contact with the high resolution image which is provided with Polaroid. If experimental work has already been carried out which will make the selection of image forming parameters quite certain, then it is possible to switch over to a recording mode using 35 mm film and to develop and print a large number of pictures later at a much reduced cost per picture.

II. Stereo-Pairs

The use of two images taken at two different aspects (tilt angle) greatly

enhances both the analytical possibilities and the experiential contact with the specimen. While such a process requires an additional expenditure in time and effort in order to obtain a second picture of the specimen, the results are most rewarding and stereo-pair production has become an essential part of scanning electron microscope operation.

1. Resolution of Analytic Ambiguities

Quantitative, three-dimensional analysis can be carried out by computer techniques and by semi-quantitative modeling techniques (BOYDE, 1970; WELLS, 1960). In addition to height measurements, it is also possible to utilize stereo-pairs to resolve questions concerning the general shape of the specimen and certain nonmetric properties such as intersection, overlay and general topological properties.

2. Enhancement of Experiential Contact

A single image produced by the secondary electron signal in a SEM utilizes depth codes of shading and overlap to impart to the observer the shape and depth characteristics necessary for experiential contact. By utilizing a second image taken at an aspect that would correspond to the position of the second eye, we can provide the information through the depth code of binocular vision. It is quite clear to anyone who has utilized a good stereoscopic viewer that the enhancement in contact, in the feeling of "being there," is considerable when this additional code is superimposed on the other two. If we are to utilize our intuitive response to systems, it is valuable to make our contact with the image as broad as possible in terms of mechanisms for viewing our environment. The addition of a stereo viewer close to the scanning electron microscope console allows the operator to add this code for depth and shape in a convenient manner.

Individual stereo viewing equipment ranges in price from $10.00 to several hundred dollars and, in general, the choice of the equipment depe.. somewhat on the range of use anticipated. For some individuals it is poss ble to assimilate a stereo-pair with a minimum of assistance from optical equipment; however, for the average viewer, a good quality stereo viewer (perhaps costing $200.00) is worthwhile if fatigue and discomfort is to be avoided and if we are to guarantee a maximum stereoscopic participation by all individuals.

3. Methods of Stereo-Pair Presentation

The presentation of stereo-pairs to more than the single viewer is usually accomplished by either slide projection or by some form of printed image. In both cases there are considerable technical difficulties present. If we consider first the presentation of stereo-pairs to a large audience means of projection, we find that a special projector is generally needed a that the audience must also be equipped with special glasses. Today, most commonly the coding necessary to separate the image for left and right eye is carried out through means of polarized light. However, other methods ha been used, notably color coding (red, green) with the same requirement for coding projector and a decoding pair of glasses for each member of the audi-. The alignment of the projected images is sometimes quite delicate and for many projection rooms it is not possible to have the alignment satisfactory for all members of the audience. It is also necessary that a special screen (lenticular screen) be used to maintain the distinction in direction of pola ization of the two images if polarized light is used.

One modification of this projection technique is to superimpose two co images on a single slide utilizing the appropriate stereoscopic viewer for proper mounting. The requirement for analyzing glasses for the audience st exists (NEMANIC, 1971) (see Appendix 2).

In terms of printing stereo-pairs in a book or journal, we are again faced with rather formidable difficulties. Several techniques have been utilized but each of these techniques have certain drawbacks. Perhaps the simplest technique for printing stereo-pairs is directed towards the somewhat selected audience that can assimilate a stereo-pair simply by looking at the two images (perhaps with the aid of a separating card between them). Such a method is very simple but obviously suffers because of the rather small audience that can be expected to achieve success with this method.

Scientific American, in their January, 1954 issue, used a mirror image printed as one of the stereo-pairs. Synthesis of the two images by the reader is achieved by placing a small mirror vertically to the page in between the two pictures.

It is also possible to print the two members of the stereo-pairs side by side and suggest to the viewer that he utilize a stereoscopic viewer to make the synthesis. If a standard design could be set for publication of the stereo-pair with respect to both size and position on the page and to the selection of a standard inexpensive stereoscopic viewer this method would become more accessible.

A ~~second~~ fourth method of printing stereo-pairs for large circulation is based on a superimposition of images as was discussed above with respect to the projection of slides. The two images can be superimposed and coded in two colors which will later be separated by the reader by means of glasses or filters. Such a presentation has been quite successful in some instances and the publishers have even considered it useful to include an inexpensive pair of viewing glasses as a part of the book or journal.

The superposition technique can also be utilized in a fashion such that the sorting of the images is accomplished by an overlay layer rather than by

requiring glasses to be worn by the reader. This technique generally makes use of small lenticular screen overlays which act as cylindrical lenses and present one of the offset images to one eye and one to the other. Such devices have been utilized to a considerable extent in the production of so-called 3D postcards. This technique, while very expensive, has been utilized for the presentation of stereoscopic information of scientific value (HARTE & RUPLEY, 1968).

It would seem that each of these methods is rather restrictive either in terms of the auxilliary equipment needed by the reader or in terms of cost. A really workable, inexpensive method for the presentation of stereo-pairs to large audiences through the media of books or journals is still needed. The vertically mounted system used in this chapter and detailed in Appendix #1 might be a step in this direction.

III. TV Tape

Rapid scan rates are useful in preliminary viewing of the stationary specimens and are essential for the observation of dynamic systems. (KIMOTO, et al., 1969) (PAWLEY & HAYES, 1971). It is sometimes useful to record these dynamic events and for this purpose the most appropriate vehicle is television tape recording. However, the difficulties of presentation of this dynamic information either in published or lecture form are considerable. As a first approach one can simply photograph the face of the TV screen in order to present one or more static samplings of the TV image. Since the function here is solely one of recording for future review or presentation rather than a dual function of recording and also integrating the image for assimilation by the observer, the types of photographic procedures are quite standard and in general would not benefit as much from the rapid processing capabilities offered by Polaroid film. We have found a Speed Graphic with a 4"x5" back quite satisfactory for this purpose.

The TV tape recording system associated with the SEM need not be expensive and it is possible to utilize nearly any of the commercially available tape recorders. However, the various brands of tape recorders are not compatible and some care must be taken to insure compatibility between the recorded tapes and the play-back recorders available at meetings or lectures.

Projection facilities in terms of adequate TV monitors throughout the audience are often lacking at scientific meetings and while there are devices which project an image from TV tape onto a screen, they are limited in terms of brightness and magnification and in our experience, have limited applicability. Such TV tape projectors may be considered only when the audience is not too large and when a sufficient number of TV tape presentations will be made to justify their cost.

A more convenient mode of presentation is to have the TV tape material transcribed onto standard 16 mm. movie film. The transcription process is rather expensive, but once the material is on 16 mm. film, presentation becomes no problem.

The static printing in journal or book form of material from TV tape fails to convey the essential quality of movement. Perhaps it will be possible in the future to distribute the tapes through a kind of "journal of video tape" but for the moment, journal presentations must rely on descriptions provided by authors and a limited number of single frame, still pictures.

F. Information Dssimilation by the Observer

The end point in our chain of information transfer which started at the specimen is the transfer of information from the image to the observer. Such information transfer might be considered as taking place along two channels. The first channel relies on the objective, analytic processes associated with our systems of ideas (physics, chemistry, biology, mathematics). It is this

analytic process that is by far the most productive and well known scientific channel. However, it has not been without its famous critics. Kierkegaard in particular has pointed out the limitations of objective reflection (KIERKEGAARD, 1848). We might explore then, the possibilities of adding subjective or experiential channels of information transfer to the more classical analytic, objective methods. First, however, let us consider the analytic methods that are available to us through SEM (JOHARI, 1971_a).

I. Analytic Information Processing

The information available in the image from the SEM might be divided on the basis of its physical characteristics into geometric information, chemical information and information related to the electrical properties of the specimen.

1. Geometric Information

a) Metric Geometry

Metric geometry deals with properties of length, angle and area and the functional relationships between these properties. This geometry is historically the oldest and still serves as the first point of contact between an observer and the analytic properties of the object being investigated.

The metric properties of a sample are invariant under translational changes but are quite sensitive to other transformations such as projection or deformation. Any study of the metric properties of a sample requires ~~is very much influenced by~~ preparative methods that ~~would~~ insure the fidelity of the angles, distances and areas involved. Also, since metric geometry by definition depends on the measured value, the resolution of the instrument is of considerable importance since it places a limit on the accuracy of measurement.

Perhaps our most common form of analysis is the familiar operation of determining geometric size and shape by measuring the lengths, angles and areas associated with the plane and solid figures represented in the scanning

electron micrograph.

Fig 10

Metric geometry is not the only geometry available for study with respect to the spatial characteristics of the specimen. Historically, projective geometry properties are next in order to be considered. Projective properties are somewhat more general than the metric geometry properties in that they are invariant not only to translation but also to projection. Under projective transformation, the properties of distance, angle and area pertinent to metric geometry do not remain constant, but there are other properties (e.g. linearity) which do remain invariant.

b) Topologic Geometry

There is a still more general set of properties which remain invariant under a wide variety of transformations. These are referred to as the topological properties and constitute a non-metric, non-projective, enumerative geometry. Such properties (e.g. genus) because of their invariance to a wide range of transformations, might be considered the more basic spatial properties of the specimen (RASHEVSKY, 1954, 1961).

The technique of studying systems by introducing controlled transformations has been most valuable in physics and mathematics and can also be applied to some extent to imaging studies (HAYES, 1972). In contrast to the usual goal of maintaining fidelity, in these studies transformations (artifacts) are purposely introduced in the hope that by sifting through specimen properties as a function of their invariance under certain transformations we may be able to extend our analytical techniques.

In general, the study of topological properties requires the ability to be able to sample information in three dimensional depth. In terms of available microscopes, the light microscope at high resolution is limited to optical "sectioning" as a result of its very narrow depth of field and the CEM by the physical restrictions of a very thin specimen. The SEM combines a very large

depth of focus with the ability to look at bulk samples. Such a sampling of intricate shapes often leads to a better appreciation of certain of the topological characteristics and to some extent can justify a loss in the metric geometry analytic capabilities as a result of the lowered resolution of the SEM (HAYES, 1970_b).

2. Chemical Information

The analysis of chemical composition has been a very useful part of light and conventional electron microscopy. Such chemical information can be presented in tabular form such as micro-photometric or electron diffraction data (GLAESER & THOMAS, 1969) or can be presented as an image such as the areas of specific stain uptake shown in the histochemical micrograph. Both numerical and imaged chemical information can be obtained from the SEM and because of the separation of probing radiation (electrons) from image forming signal (x-rays, Auger electrons, visible light, etc.) the SEM offers some unique advantages in this area (JOHARI, 1971_b).

a) Characteristic X-Ray Elemental Analysis

The electron microprobe for many years has utilized the characteristic x-rays emitted from a sample bombarded by electrons to identify and quantitate the specimens elemental composition (ANSELL, 1969); (KNASTON & STEWART, 1969). These x-rays originate from the excited electron shells (K, L, M, etc.) of the specimen atoms and the x-ray wavelength or energy is characteristic for each element. The characteristic x-ray spectra in general cannot be used to determine which chemical bonds exist between these atoms.

For biological applications, the usefulness of an x-ray attachment for the SEM depends largely on the value of elemental analysis to the biological researcher. While there are some examples of biological systems which are very sensitive to the concentration of a particular element, it is more the general rule that biology is more concerned with the chemical relationships between

fairly common atoms. It is possible that as we limit the area studied by utilizing the very fine probing beam of the SEM (SUTFIN, et al., 1971), we may find locally high concentrations of unusual elements but most often we are dealing with very low concentrations of all but the common light elements. Elemental micro-analysis by the SEM (ANSELL AND JUDD, 1969) (RUSS, 1971) should be studied with the same attention to questions of sensitivity (elemental concentration which can be measured), matrix interference, specimen geometry and counting statistics that would be necessary before applying the electron microprobe (BROWN, et al., 1971); GARDNER and HALL, 1969) or x-ray fluorescence techniques to the problem.

The ability to form an SEM (or microprobe) image using the characteristic x-ray signal is also available and offers an additional way that the observer can understand the relationship of the chemical elements within the specimen (RUSS, 1971; TOUSIMIS, 1969).

b) Auger Spectra

Energies of the Auger electrons ejected from the specimen are also characteristic of the elemental composition but the Auger yield for the light elements ($2<Z<15$) is high compared to the characteristic x-ray emission in this region (MAC DONALD, 1971). An analysis of the Auger spectra also gives an indication of the discrete location of the element below the surface of the specimen. The short range of the Auger electron compared to the corresponding characteristic x-ray allows depth analysis by Auger electron spectroscopy in the 1 to 10 Å range. This very short range also means that the volume analyzed is very much smaller than the volume analyzed by characteristic x-ray techniques. This could be of considerable importance to the biological investigator if certain elements exist in reasonably high concentrations only in very small domains.

c) Cathodoluminescence Analysis

Certain materials will emit visible light when bombarded with electrons. This cathodoluminescence can be counted and used as the information signal in

the SEM. This mode of operation provides information concerning the chemical composition of the specimen at a more complex level than elemental analysis. Cathodoluminescence is more related to the molecular and solid-state properties of the specimen.

It would be of great advantage in biological research to use a variety of specific cathodoluminescent stains to locate these chemical entities but, although some work in this direction has been partially successful (PEASE & HAYES, 1966), the problems of photon yield, poisoning of the stain and electron radiation damage remain a considerable obstacle (PEASE, 1967).

d) Energy-loss Spectra

As electrons pass through a thin specimen in the scanning transmission mode of operation, they lose energy in a way that is characteristic of the composition of the specimen. If the energy loss peaks are analyzed, a great deal of useful information can be extracted, some of which corresponds to the information contained in the analysis of several of the excitation modes. Crewe and co-workers have developed techniques of energy loss spectroscopy (CREWE, et al., 1966) and combined them with very high resolution scanning transmission microscopy to produce chemical contrast in images and quantitative data describing composition (CREWE, et al., 1970) (CREWE, 1971). The ability to utilize more of the in-elastically scattered electrons as well as elastically scattered electrons improves the image characteristics of this scanning transmission system as compared to conventional electron microscopy. Such advantage may also allow the examination of thick sections (SWIFT & BROWN, 1970) at very much lower accelerating potential than the ultra high voltage conventional electron microscopes used for this purpose.

3. Electrical Properties and Charging

One of the earliest and most prominent areas of application of the SEM is the study of the electrical properties of inorganic materials and electronic circuits. The net number of electrons gained by the specimen (specimen current) or, more commonly, the changes produced in a current flowing in the specimen (induced specimen current) is used as the video signal for the scanning

electron micrograph (EVERHART, et al., 1964; EVERHART, 1966). Direct applications of these modes of operation to biological samples has been small but the electrical properties of the specimen and their interaction with the electron beam is of considerable importance to biologists as it affects the artifact of charging (PAWLEY, 1972a,c; EVERHART, 1970; BOYDE, 1971). This commonly encountered artifact can only be properly recognized if the observer is aware of the factors that influence secondary and backscattered electron emission, collection efficiency and surface potential.

II. Experiential or Subjective Information Processing

In addition to its capabilities as an analytical tool, the SEM has some obvious advantages in presenting information that can be experienced directly.

1. Models of Perception

If we are to construct instrumentation that can extend our senses, it is useful to consider some models of visual perception that might be applied to the microscopic world under investigation. *Arnheim* has suggested three models of perception based on an interpretation of the relationship between an object and its environment (ARNHEIM, 1969): (1) The first mode of perception does not distinguish between the properties associated with the object and those properties contributed by the environment. It can be represented by the camera or "peep-hole" approach. Each small unit of the image is considered independently of its surroundings and no distinction is attempted between essential characteristics and accidental attributes. In such a perceptive attitude, the principal criteria is fidelity between image and object. (2) The second model of perception suggested by *Arnheim* might be characterized as the absolute scientific approach. In such an approach the object is stripped of all "non-essential" properties and only the abstracted values relating to a predetermined essence of the object are considered. This kind of approach has shown itself to be very valuable as a scientific technique where the action of the envir-

onment may be considered as artifactual and influencing the image in a distortive way. (3) The third model of perception uses the environment to reveal properties of the specimen that are not readily apparent in any one static environment. In art, this model might be represented by the Impressionist school of painting. Environmental factors such as lighting are observed under changing conditions in order that the qualities of the object are more clearly revealed. Environment becomes a tool for the study of the object. At various levels the invariance to certain transformations reflect fundamental characteristics of the specimen that would not be apparent under static nonvariant conditions.

Instead of treating all artifacts or deformations as negative or unproductive, we might be able to utilize these same factors as an additional way of probing for the characteristics of the specimen.

2. Limits of Analytical Information Processing

The transfer of information by visual pathways is not limited to analytic processes; an artist through his painting transfers large amounts of information about himself and his view of the world by methods which often resist the analytic process. Many of the same techniques that have been so successful in visual art could be attempted in viewing the microscope world if instrumentation techniques could be developed which allows the individual observer to contact the specimen through channels which are similar to the ways in which he views his own environment.

Often the kinds of things that we learn through such subjective interaction are not easy to describe. Our understanding of the object viewed seems to be increased in a general way but specific statements about the type of learning which we have experienced are difficult to make. About all we can say is that it seems to be valuable to be open to the possibilities of subjective, experien-

tial contact and to be free to make use of feelings and ideas that such contact may inspire.

The analytic, objective approach has protected the observer from unrecognized artifact, image aberrations, misleading display modes and personal bias. But it has also been restrictive in that objectivity and analysis tend to remove the observer as a person from the investigation (KIERKEGAARD, 1848) and in his place substitute a very limited system of ideas.

3. Possibilities of Complementary Subjective and Analytic Investigations

It would seem useful for the observer to be able to move comfortably between the objective and subjective methods. An over-strict adherence to objectivity can be severely limiting to the general understanding and appreciation of the specimen. On the other hand, an over indulgence in subjectivity leaves the observer adrift in a sea of emotions.

It is true that a scanning electron microscopist may be overly impressed with simply "pretty pictures" but there is an equal danger that our respect for beauty as a powerful and respectable criterion in our search for methods of transferring information may be seriously weakened if we pay too much attention to the cries for objectivity heard so often. Perhaps it is not necessary to choose between artistic methods and scientific methods; between subjective and objective approaches (HAYES, 1969), (HAYES, 1971a), (HAYES, 1971b). If we can operate with an objectivity which is free of personal bias and also apply a subjectivity which is independent and unafraid of the criticism of our peers, we can bring to the attack on the problem not only the best of our systems, but the best of ourselves.

G. Conclusion

I would like to pose some questions that might face the biological scientist as he considers the possibilities for scanning electron microscopy in

Questions Regarding a Scanning Electron Microscope Program for Biological Study

his own field.

1. Is the SEM Really Necessary? This question can only be answered after carefully considering both the kinds of information that are required from the microscope system and also the possible alternative systems which are available. Often the technique of replication (either directly from the specimen or after freeze-etching procedures) would be the alternative of choice (KOEHLER, 1968; STEERE, 1969_a; 1971_b). The increased resolution (STEERE, 1971_a) and the ability to provide a method for reaching deeper-lying structures while still preserving the integrity of the biological system (KOEHLER, 1967; STEERE, 1969_b) makes this technique very attractive.

One should also keep in mind that by using careful microscopic techniques which utilize visible light radiation, a great deal of information can be obtained at resolutions which often are quite adequate for the particular system under investigation. Differential interference microscopy (Nomarski) (ALLEN et al., 1969) and the procedures of phase microscopy may result in a satisfactory acquisition of the needed information (KAYDEN and BESSIS, 1970; BAJER and ALLEN, 1966) from the specimen without the very significant capital outlay associated with scanning electron microscopy. In addition, the particular advantages in terms of a hydrated system and the interaction of visible light with matter may, in fact, allow the light microscope to cover areas not available to SEM. Before resorting to scanning electron microscopy, a rather clear decision is necessary that the particular needs of the experiment cannot be met through the use of the more traditional forms of microscopy (KOEHLER, 1971).

2. Which Instrument? Several commercial brands and sizes of scanning electron microscopes are now available and each manufacturer has several models or accessory packages. Each of these instruments offers certain unique features and the selection of a particular brand requires generally a

rather close contact with the actual operating instrument. It is most often necessary for the investigator to observe his own sample material in several of the instruments in order to try to appreciate which instrument will be best for particular use. The kind of sample that will be used, the number of different operators that may use the microscope, the facilities for maintenance and repair that are available to the prospective buyer all must be considered when selecting an instrument. Special stage requirements, a variety of signal collection systems (X-ray, cathodoluminescence, etc.), TV scan rate, are also often of prime importance to particular investigations.

In addition to primary scanning electron microscopes, there are also electron microscopes which can operate in both the conventional and the scanning modes. Such instrumentation, particularly with respect to scanning transmission operation (SWIFT & BROWN, 1970), can be quite effective and offer (after a moderate amount of modification), the capability of high resolution conventional electron microscopy coupled with a useful flexibility in terms of both secondary electron image and the transmitted electron signal.

The choice of instruments is not a simple one and requires that the investigator visit the demonstration facilities of several manufacturers; carrying with him his own sample and even preferably operating the instrument himself. It is only by making such a thorough investigation that the advantage of the different instruments to any one program can be accurately determined.

3. What Auxilliary Equipment Might be Needed? Nearly all SEM investigations are greatly enriched by a broad correlative study in other disciplines (FORRESTER, et al., 1969b) (LEWIS, 1971). In particular, light microscope and conventional electron microscope facilities provide information which makes the scanning electron micrograph very much more useful (KOEHLER & HAYES, 1969a) (KOEHLER & HAYES, 1969b) (MC DONALD & HAYES, 1969) (MC ALLISTER &

HADEK, 1970). An SEM isolated from these facilities, particularly in biological research, would be difficult to use to its full potential. The addition of multiple microscope techniques yields a final output which is considerably greater than the sum of its parts and close cooperation between light microscopists, conventional electron microscopists and scanning electron microscope investigators (GEISSINGER, 1971) (MC ALEAR, et al., 1967) (MC CALLISTER & HADEK, 1970) is very desirable.

There are also several units of auxilliary SEM equipment that are generally useful: 1) A binocular, stereoscopic light microscope. The SEM is often used as an extension of a dissecting or stereoscopic light microscope and the scanning facility should include the very best instrument of this type. The advantages in terms of orienting the observer to the specimen by means of this microscope's relatively large field of view and its use in the preparation and mounting of specimens justifies the acquisition of an excellent light microscope of this type. 2) Freeze-dryer. Freeze-drying is probably the most often utilized method of removing the water from biological tissues with a minimum of artifact. The freeze-dryer utilized should have monitoring and control of both temperature and vacuum systems and should dry the sample with a minimum of contamination. 3) Critical-point dryer. Critical-point drying represents an excellent alternative method of water removal and drying by this technique is a very valuable check on freezing artifacts possibly introduced during freeze-drying. A critical-point dryer that is capable of utilizing either carbon dioxide or the freons as the transition fluid is desirable. 4) Vacuum evaporator. The vacuum evaporator is utilized as the common method of placing a conducting layer on the sample. Considerations of minimum contamination are important and the ability to rotate (and possibly tilt) the sample is necessary if a uniform conducting layer is to be achieved. It is often necessary to

evaporate several different kinds of material without breaking the vacuum and therefore multiple electrodes are desirable. 5) Stereo viewer. An optical stereo viewer placed near the microscope allows the operator to view stereo-pairs conveniently and has become a very much used part of the SEM laboratory equipment. The viewer should be flexible enough to provide comfortable viewing for different operators. 6) Signal monitor oscilloscope. The monitoring of the video signal and the measurement of the final light production on the display cathode ray tube is very valuable in the recording of images and in trouble shooting the microscope. It is generally worthwhile to establish a permanent signal monitoring facility with sufficient capabilities to carry out both of these functions effectively. 7) TV tape recorder. For any dynamic study, it is necessary that the information be stored for review and analysis on video tape. The video tape recorder need not be elaborate and a variety of suitable models are on the market. If other video facilities are near by, the question of compatibility between video recorders might be a consideration.

II. Prospects for the Future

Imaging in the secondary electron mode as an aid to recognition of shape and structure will probably remain the central use of the instrument. The resolution in this mode has improved with the addition of high intensity guns and further improvement can be expected at least for specialized modes of operation such as scanning transmission. The X-ray and cathodoluminescent modes have been less well explored in terms of biological applications and their use in an histochemical analysis will become more common in the future.

It would seem that the smaller specialized instruments available at lower cost will prove valuable in many apsects of biological research. Small scanning electron microscopes can now be designed in a cost range comparable to a

conventional electron microscope and a very limited scanning system might be constructed in the future for a price comparable to that of a good light microscope. Such simple systems, of course, would necessarily be limited in their performance, but it may be found that the operations that they can perform are the ones needed in many biological investigations. If this proves to be the case, we might find that scanning systems can be located in individual biological laboratories rather than in a central facility, and that they can be available to the individual investigator in a way similar to the light microscope.

We must resist the temptation to say that a new instrument is automatically a better instrument. The uses of the SEM in biology are relatively specific and narrow as compared to the light microscope or even the conventional electron microscope. However, it seems that already the position of scanning electron microscopy, as a useful additional tool for the investigation of biological structure, is assured.

Acknowledgments

The author would like to acknowledge the generous support of the Electronics, Mechanical and Graphic Arts groups at Donner Laboratory and Lawrence Berkeley Laboratory. A special acknowledgment belongs to Dr. James B. Pawley who has contributed so much to the SEM program at Donner. His instrumentation design, experimental results and critical comments have been of great value during the preparation of this chapter. My thanks also to Ms. Dorothy Sprague for her expert help in preparing the manuscript and to Dr. James Koehler, University of Washington, for his continued interest in this project.

H. Appendices

I. Optical Aids for the Viewing of Vertically Mounted Stereo-Pairs

1. Lucite Prism.

A small prism can be held in front of one eye in order to deflect the image of one of the stereo-pairs making this image coincide with the image of the second member of the stereo-pair which is viewed directly with the other eye. The amount of deflection is controlled by adjusting the distance between the page of the book and the prism. Problems of convergence, inter-ocular distance and picture size are reduced by utilizing vertical rather than horizontal mount- of the stereo-pairs.

When the prism to picture distance is properly adjusted, the observer will see three images: a monocular image above and below the coincident stereoscopic image which is in the center.

The prism can be constructed by cutting a wedge of lucite of about 25° pitch and faces of perhaps 2" x 2". The faces are then polished until a satisfactory clarity is obtained. Such a prism will provide the appropriate deflection when held approximately 12 inches from the page of the book. We have found it convenient to hold the prism about 2" in front of the right eye with the apex pointing towards the observer, and the bottom surface parallel to the plane of the book.

2. Microscope Slide — Corn Syrup Prism.

A simple and inexpensive prism can be constructed using standard 1" x 3" light microscope slides to form the wedge and liquid corn syrup as the refracting material. The long edges of two slides are held tightly together and placed on the center section of a 5½" strip of ½" wide adhesive tape. The tape will stick to the bottom edges of the slides and can be picked up and pressed against the outer faces of the slides, sealing the long bottom edge. The top edges of the slides are now separated to a position a little less than ½" apart forming an

open wedge. Holding the slides in this position the strip of tape is brought up the ends of the wedge. The tape now forms the end covers of the wedge.

The wedge is now filled with clear corn syrup (Karo, light) and the top is sealed with a second strip of ½" adhesive tape.

Such liquid prisms tend to be self-sealing as the corn syrup might be exposed to air and the prisms retain good optical properties over many months time with no appreciable leaking or drying.

The finished prism is held about 2" in front of the right eye with the apex pointing down and the distance from prism to page is adjusted until coincidence of the left and right eye image occurs (see above).

3. L. A. Mannheim has reported that two small plastic prisms have been incorporated into a pair of glasses suitable for commercial manufacture(MANNHEIM, 1971). The price of these glasses would be in the range of $3.00 to $6.00 and they are the product of Stereo Vertrieb Nesch, 44 Munster, Enschedewey 78, West Germany.

4. Two small mirrors(purse mirrors) can also be used to accomplish the vertical image displacement. In contrast to the single prism method, two mirrors are necessary, one for each eye, in order to prevent one eye seeing a mirror image and the other a direct image. The two mirrors are held against the eyebrows at a 45° angle so that the observer looks straight ahead and sees the page of the book directly beneath the mirrors. The mirrors are now rotated slightly producing vertical displacement of one member of the stereo-pair with respect to the other until the image for the left eye and the image for the right eye coincide to give a stereoscopic display.

II. Projection of Stereo-Pairs by Means of a Superimposed Color-Coded Transparency ($3\frac{1}{4}$" x 4" lantern slide, standard projector and screen).

M. Nemanic, University of California, Berkeley

1. Original Micrographs. Take normal black and whites on No. 107 Polaroid film with 7° of tilt between the pair.

   a) focus using the z-axis control;

   b) center the same area in both micrographs.

2. Framing for Superposition. Use a Polaroid MP-3 Industrial Viewer, a graflex 120 roll film back and a framing table.

   a) place the black and white print in the framing table; center the micrograph by viewing through the camera lens; enlarge the image to fill the format of the roll film back;

   b) place Ectacolor or Ektachrome color film in the back.

3. Color Coding of the Two Superimposed Images.

   a) use Wratten 25a (red) and 58 (green) gelatin filters;

   b) exposure settings:

		Speed	f
Ectacolor Type S (ASA 100)	Green:	1/2	4.7
(for negative slides)	Red:	1/8	5.6
High Speed Ektachrome (ASA 160)	Green:	1/2	5.6
(for positive slides)	Red:	1/8	8

   c) standardize the use of red or green for the lower angle micrograph;

   d) expose the first micrograph of the stereo-pair with the appropriate filter over the lens (hand-held);

   e) place the second micrograph of the pair in the same frame as the first without advancing film; set speed and shutter opening for the new filter; expose, producing

double exposed superposition and then advance the film for the next pair.

4. Processing and Mounting.

a) standard color processing is carried out by the photo-lab or by a commercial camera shop;

b) the developed film is mounted between lantern slide glass in the usual way.

5. Viewing.

a) red and green acetate, found in an art supplies store, can be used to make simple viewers for large groups of people. Two thicknesses of green and three of red approximate the color and optical density of filters used to make the color composite. Small squares are held in front of each eye while viewing the projection;

b) instruct viewers as to the correct color filter for right and left eye, depending on convention adapted in 3(c) above.

LITERATURE

ALLEN, R. D., G. B. DAVID and G. NORMARSKI: The Zeiss-Nomarski differntial interference equipment for transmitted-light microscopy. Z. wiss. Mikr. u. Mikr. Tekn. 69, 193-221 (1969).

ANDERSON, T. F.: Techniques for the Preservation of Three-Dimensional Structure in Preparing Specimens for the Electron Microscope. Trans N. Y. Acad. Sci. 13, 130-134 (1951).

ANSELL, G. S. and G. JUDD: Thin Film Electron Probe -- Electron Microscope Microanalysis Techniques in Biomaterials Studies. Trans. N. Y. Acad. Sci., Ser. II, 31, 637-647 (1969).

AREMBERG, I. K., W. F. MAROVITZ and A. P. MAC KENZIE: Preparative Techniques for the Study of Soft Biologic Tissues in the Scanning Electron Microscope. Trans. Amer. Acad. Ophthal. & Otolaryng. 75, 1333-1345 (1971).

ARNHEIM, R.: Visual Thinking, 1-345, Univ. of Calif. Press, Berkeley, and Los Angeles 1969.

ARNOLD, J. D., A. E. BERGER and O. L. ALLISON: Some Problems of Fixation of Selected Biological Samples for SEM Examination. In: Scanning Electron Microscopy, Johari, O., ed., 249-256. I.I.T. Research Institute, Chicago, Illinois 1971.

BAJER, A. and R. D. ALLEN: Structure and Organization of the Living Mitotic Spindle of Haemanthus Endosperm. Science 151, 572-574 (1966).

BAKER, J. R.: Principles of Biological Microtechnique, 1-357, Methuen & Co., London 1958.

BAKER, R. F.: Ion Etching of Red Blood Cells. In: Red Cell Membrane Structure and Function, Jameson, G. A. and Greenwalt, T. J., Eds.: 13-35. J. B. Lippincott Co., Philadelphia 1969.

BARBER, V. C. and A. BOYDE: Scanning Electron Microscopic Studies of Cilia, A. Zellforsch., 84, 269-284 (1968).

BESSIS, M. and L. S. LESSIN: The Descocyte-Echinocyte Equilibrium of the Normal and Patologic Red Cell. Blood 36, 399-403 (1970).

BLUMKE, S. and K. MORGENROTH: The Stereo Ultrastructure of the External and Internal Surface of the Cornea. J. Ultrastruct. Res. 18, 502-518 (1967).

BOK, A. B., J. B. LE POOLE, J. ROOS and H. DE LANG: Mirror Electron Microscopy. In: Adv. in Optical and Electron Microscopy, Barer, R. and Cosslett, V. E., Eds.; Vol. 4, 161-383, Academic Press, N. Y., 1971.

BOYDE, A. and A. D. G. STEWART: A Study of the Etching of Dental Tissues with Argon Ion Beams. J. Ultrastruct. Res. 7, 159-172 (1962).

BOYDE, A.: The Development of Enamel Structure. Proc. Roy. Soc. Med. 60, 923-928 (1967).

BOYDE, A. and J. C. P. WILLIAMS: Surface Morphology of Frog Striated Muscle as Prepared for and Examined in the Scanning Electron Microscope. J. Physiol. 197, 10P-11P (1968).

BOYDE, A., D. W. JAMES, R. L. TRESMAN and R. A. WILLIS: Outgrowth from Chick Embryo Spinal Chord in vitro Studied with the Scanning Electron Microscope. Z. Zellforsch. 90, 1-18 (1968).

BOYDE, A., F. GRAINGER and D. W. JAMES: Scanning Electron Microscopy of Chick Embryo Fibroblasts in vitro, with Particular Reference to the Movement of Cells Under Others. Z. Zellforsch. 94, 46-55 (1969).

BOYDE, A., and C. WOOD: Preparation of Animal Tissues for Surface-Scanning Electron Microscopy. J. Microscopy 90, 221-249 (1969).

BOYDE A.: Practical Problems and Methods in the Three-Dimensional Analysis of Scanning Electron Microscope Images. In: Scanning Electron Microscopy, Johari, O., Ed.; 105-112. I.I.T. Research Institute, Chicago, Illinois 1970.

BOYDE, A.: A Review of Problems of Interpretation of the Scanning Electron Microscope Image with Special Regard to Methods of Specimen Preparation. In: Scanning Electron Microscopy, (Johari, O., Ed.; 1-8, I.I.T. Research Institute, Chicago, Illinois 1971.

BRAGGINS, D. W., G. M. GARDNER and D. W. GIBBARD: The Applications of Image Analysis Techniques to Scanning Electron Microscopy. In: Scanning Electron Microscopy, (Johari, O., Ed.), 393-400. I.I.T. Research Institute, Chicago, Illinois 1971.

BROERS, A. N.: A New High Resolution Reflection Scanning Electron Microscope. Rev. Sci. Instr. 40, 1040-1045 (1969).

BROERS, A. N.: Factors Affecting Resolution in the Scanning Electron Microscope. In: Scanning Electron Microscopy, Johari, O., Ed.; 1-8. I.I.T. Research Institute, Chicago, Illinois 1970.

BROWN, A. C., R. J. GERDES and J. JOHNSON: Scanning Electron Microscopy and Electron Probe Analysis of Congenital Hair Defects. In: Scanning Electron Microscopy, Johari, O., Ed.; 369-376. I.I.T. Research Institute, Chicago, Illinois 1971.

BUDINGER, T. F.: Transfer Function Theory and Image Evaluation in Biology: Applications in Electron Microscopy and Nuclear Medicine. University of California, Berkeley, Lawrence Berkeley Lab. Report, LBL, 565, 232 pp., 1971.

BURKE, J. F., INDEBETOUW, G. NOMARSKI, G. W. STROKE: White-light Three Dimensional Microscopy Using Multiple-image Storing and Decoding. Nature 231, 303-306 (1971).

BYWATER, N. E. and T. BUCKLEY: A New Technique for Specimen Manipulation in the Scanning Electron Microscope. J. Microscopy 92, 113-118 (1970).

CARR, K. E.: Applications of Scanning Electron Microscopy in Biology. In: Int. Rev. Cyt., Bourne, G. H. and Danielli, J. F., Eds.; Vol. 30, 183-255. New York: Academic Press, ~~N. Y.~~ 1971.

CLARKE, J. A. and A. J. SALSBURY: Surface Ultramicroscopy of Human Blood Cells. Nature, 215, 402-404 (1967).

COHEN, A. L., D. P. MARLOW and G. E. GRANER: A Rapid Critical Point Method Using Fluorocarbons("Freons") as Intermediate and Transitional Fluids. J. de Microscopie 7, 331-342 (1968).

COSSLETT, V. E.: Modern Microscopy, 160 pp. G. Bell and Sons, Ltd., London, 1966.

CREWE, A. V.: Scanning Electron Microscopes: Is High Resolution Possible? Science 154, 729-738 (1966).

CREWE A. V., M. ISAACSON, and D. JOHNSON: A Simple Scanning Electron Microscope. Rev. Sci. Instr. 40, 241-246 (1969).

CREWE, A. V., J. WALL, and J. LANGMORE: Visibility of Single Atoms. Science, 168, 1338-1340 (1970).

CREWE, A. V.: A High Resolution Scanning Electron Microscope. Scientific American 224, (4) April, 26-35 (1971).

CROWE, J. H. and A. F. COOPER: Cryptobiosis. Scientific American 225, (6), December, 30-45 (1971).

DARRAGH, P. J., A. J. GASKIN, B. C. TERRELL, and J. V. SANDERS: Origin of Precious Opal. Nature 209, 13-16 (1966).

DORFLER, G. and J. C. RUSS: A System for Stereometric Analysis with the Scanning Electron Microscope. In: Scanning Electron Microscopy, Johari, O., ed., 65-72. I.I.T. Research Institute, Chicago, Ill. 1970.

ECHLIN, P.: The Examination of Biological Material at Low Temperatures. In: Scanning Electron Microscopy, Johari, O., Ed., 225-232. I.I.T. Research Institute, Chicago, Illinois 1971.

ELIAS, HANS: Three-Dimensional Structure Identified from Single Sections. Science 174, 993-1000 (1971).

ERBEN, H. K.: Application of the Scanning Electron Microscope in Paleobiology. In: Scanning Electron Microscopy, Johari, O., Ed., 233-239. I.I.T Research Institute, Chicago, Illinois 1970.

EVERHART, T. E., O. C. WELLS, and C. W. OATLEY: Factors Affecting Contrast and Resolution in the Scanning Electron Microscope. J. Elect. Control 7, 97-111 (1959).

EVERHART, T. E., O. C. WELLS and R. K. MATTA: Evaluation of Passivated Integrated Circuits Using the Scanning Electron Microscope. J. Electrochem. Soc. 111, 929-936 (1964).

EVERHART, T. E.: Deflection-Modulation CRT Display. Proc. Inst. Elect. Electron. Engrs. 54, 1480-1482 (1966).

EVERHART, T. E.: Reflections on Scanning Electron Microscopy. In: Scanning Electron Microscopy, Johari, O., ed., 1-12. I.I.T. Research Institute, Chicago, Illinois 1968.

EVERHART, T. E.: Contrast and Resolution in the Scanning Electron Microscope. In: Proc. 3rd Amer. Stereoscan Colloquium, 1-8. Kent Cambridge Scientific, Inc., Morton Grove, Illinois 1970.

EVERHART, T. E. and T. L. HAYES: The Scanning Electron Microscope. Scientific American 226, No. 1, 54-69 (1972).

FORRESTER, J. C., T. L. HAYES, R. F. W. PEASE, and T. K. HUNT: Scanning Electron Microscopy of Healing Wounds. Nature (Lond.) 221, 373-374 (1969a).

FORRESTER, J. C., B. H. ZEDERFELDT, and T. K. HUNT: The Tape-Closed Wound -- A Bioengineering Analysis. J. Surg. Res. 9, 537-542 (1969b).

FRANK, J.: Computer Processing of Electron Micrographs, in this volume, 1972. J. Koehler, Ed.

GARDNER, D. L. and T. A. HALL: Electron-microprobe Analysis of Sites of Silver Deposition in Avian Bone Stained by the v. Kossa Technique. J. Path. 98, 105-109 (1969).

GEISSINGER, H. D.: Correlated Light Optical and Scanning Electron Microscopy of Gram Smears of Bacteria and Parafin Sections of Cardiac Muscle. J. Microscopy 93, 109-117 (1971).

GLAESER, R. M. and G. THOMAS: Application of Electron Diffraction to Biological Electron Microscopy. Biophys. J. 9, 1073-1099 (1969).

GOLOMB, H. M. and G. F. BAHR: Scanning Electron Microscope Observations on Surface Structure of Isolated Human Chromosomes. Science 171, 1024-1026 (1971).

HAGGIS, G. H.: Cryofracture of Biological Material. In: Scanning Electron Microscopy, Johari, O. Ed.; 97-104. I.I.T. Research Institute, Chicago, Illinois 1970.

HARTE, R. A. and J. A. RUPLEY: Three-Dimensional Pictures of Molecular Models --Lysozyme. J. Biol. Chem. 243, 1663-1669 (1968).

HARTMAN, H. and T. L. HAYES: Scanning Electron Microscopy of Drosophila. J. Hered. 62, 41-44 (1971).

HAYES, T. L., R. F. W. PEASE and L. W. MC DONALD: Applications of the Scanning Electron Microscope to Biologic Investigations. Lab. Invest. 15, 1320-1326 (1966).

HAYES, T. L., and R. F. W. PEASE: The Scanning Electron Microscope: Principles and Applications in Biology and Medicine. In: Advances in Biological and Medical Physics, Vol. 12, Lawrence, J. H. and Gofman, J. W., Eds.; 85-137, Academic Press, Inc., N. Y. 1968.

HAYES, T. L., R. M. GLAESER and J. B. PAWLEY: Information Content of Electron Microscope Images: Color and Depth Information as Vehicles for Subjective Information Transfer. In: Proc. 27th Ann. Meeting Electron Microscopic Soc. Amer., Arceneaux, C., ed., 410-411. Claitor's Publishing, New Orleans 1969.

HAYES, T. L.: The Scanning Electron Microscope: A High Information Content Image of Biological Systems. Int. Convention, Inst. Elect. Electron. Engrs. Digest, 158-159. I.E.E.E., New York (1969).

HAYES, T. L. and R. F. W. PEASE: The Scanning Electron Microscope: A Non-focused, Multi-information Image. In: Data Extraction and Processing of Optical Images, Tolles, W. E., Ed.; 157, 497-509. Annals N.Y. Acad. Sci., 1969.

HAYES, T. L.: The Penetrating Eye -- Biomedical Applications of the Scanning Electron Microscope. 21 min. sound 16mm film, Medical Communications Division, Lilly Research Laboratories, Indianapolis, 1970_a.

HAYES, T. L.: Some Comments on the Imaging of Biological Systems. Int. Convention Inst. Elect. Electron. Eng. Digest, 154-155. I.E.E.E., New York (1970_b).

HAYES, T. L.: Microvision. In: Scanning Electron Microscopy, Ed.: Johari, O., ~~ed.~~, 521-528. I.I.T. Research Institute, Chicago, Illinois 1971_a.

HAYES, T. L.: The Scanning Electron Microscope: Multi-Radiation, Non-focused Imaging. In: Advances in Medical Physics, Laughlin, J. S., Ed.; 124-140. 2nd Int. Conference Med. Phys. Inc., Massachusetts General Hospital, Boston, 1971_b.

HAYES, T. L.: Invariance Under Transformation: A Useful Concept in the Interpretation of SEM images. In: Scanning Electron Microscopy, Johari, O., Ed.; I.I.T. Research Institute, Chicago, Illinois, in press 1972.

HEINRICH, K. F. J., C. FIORI and H. YAKOWITZ: Image-Formation Technique for Scanning Electron Microscopy and Electron Probe Microanalysis. Science 167, 1129-1131 (1970).

HESLOP-HARRISON, Y. and J. HESLOP-HARRISON: Scanning Electron Microscopy of Leaf Surfaces. In: Scanning Electron Microscopy, Johari, O., ed., 117-126. I.I.T. Research Institute, Chicago, Illinois 1969.

HILLMAN, D. E. and E. R. LEWIS: Morphological Basis for a Mechanical Linkage in Otolithic Receptor Transduction in the Frog. Science 174, 416-419 (1971).

HORRIDGE, G. A. and S. L. TAMM: Critical Point Drying for Scanning Electron Microscope Study of Ciliary Motion. Science 163, 817-818 (1969).

HUMPHREYS, W. J., T. L. HAYES and R. F. W. PEASE: Transmission and Scanning Electron Microscopy of Cryptobiotic Cytoplasm. In: Proc. 25th Ann. Meeting Electron Microscope Soc. Amer., Arceneaux, C., ed., 50-51. Claitor's Publishing, New Orleans 1967.

JAQUES, W. E., J. COALSON and A. ZERVINS: Application of the Scanning Electron Microscope to Human Tissues: A Preliminary Study. Exp. Molec. Path. 4, 576-580 (1965).

JOHARI, O.: SEM and Analytical Possibilities. In: Scanning Electron Microscopy, Johari, O., ed., 529-536. I.I.T. Research Institute, Chicago, Illinois, 1971_a.

JOHARI, O.: Total Materials Characterization with the Scanning Electron Microscope. Research/Development 22, 12-20 (1971_b).

JONES, FRANCIS: The Use of Opal as an SEM Standard, Personal Communication, (1969).

JONES, J. B., J. V. SANDERS and E. R. SEGNIT: Structure of Opal. Nature (Lond.) 204, 990 (1964).

KARNOVSKY, M. J.: A Formaldehyde-Glutaraldehyde Fixative of High Osmolarity for Use in Electron Microscopy. J. Cell. Biol. 27, 137A-138A (1965).

KAYDEN, H. J. and M. BESSIS: Morphology of Normal Erythrocyte and Acanthocyte Using Normarski Optics and the Scanning Electron Microscope. Blood 35, 427-436 (1970).

KIERKEGAARD, S.: Concluding Unscientific Postscript, 1848. Translated by David F. Swenson and Walter Lowrie. Princeton University Press 1941.

KIMOTO, S., H. HASHIMOTO and S. TEKASHIMA: Transmission Scanning Microscope. In: Scanning Electron Microscopy, Johari, O., Ed., 81-88. I.I.T. Research Institute, Chicago, Illinois 1969.

KIMOTO, S., M. SATO and T. ADACHI: TV Scanning Device in a Scanning Electron Microscope and Its Applications. In: Scaning Electron Microscopy, Johari, O., Ed., 65-72. I.I.T. Research Institute, Chicago, Illinois 1969.

KNASTON, D. and A. D. G. STEWART: Composition Analysis in the Stereoscan. In: Scanning Electron Microscopy, Johari, O., Ed., 465-470. I.I.T. Research Institute, Chicago, Illinois 1969.

KOEHLER, J. K.: Studies in the Survival of the Rotifer Philodina after Freezing and Thawing. Cryobiology 3, 392-399 (1967).

KOEHLER, J. K.: The Technique and Application of Freeze-Etching in Ultrastructure Research. In: Advances in Biological and Medical Physics, Lawrence, J. H. and Gofman, J. W., Eds., Vol. 12, 1-84. Academic Press, Inc., New York: 1968.

KOEHLER, J. K. and T. L. HAYES: The Rotifer Jaw: A Scanning and Transmission Electron Microscope Study, I. The Trophi of Philodina acuticornis odiosa. J. Ultrastruct. Res. 27, 402-418 (1969a).

KOEHLER, J. K. and T. L. HAYES: The Rotifer Jaw: A Scanning and Transmission Electron Microscope Study, II. The Trophi of Asplanchna sieboldi. J. Ultrastruct. Res. 27, 419-434 (1969b).

KOEHLER, J. K.: Comparative Examination of Biological Samples by SEM, TEM and Other Techniques. In: Scanning Electron Microscopy, Johari, O., Ed.: 241-248. I.I.T. Research Institute, Chicago, Illinois 1971.

KRINSLEY, D. H. and P. W. HYDE: Cathodoluminescence of Studies of Sediments. In: Scanning Electron Microscopy, Johari, O., Ed.: 409-416. I.I.T. Research Institute, Chicago, Illinois 1971.

KUWABARA, T.: Surface Structure of the Eye Tissue. In: Scanning Electron Microscopy, Johari, O., Ed.: 185-192. I.I.T. Research Institute 1970.

LANGMUIR, D. B.: Theoretical Limitations of Cathode-Ray Tubes. Proceedings of the Institute of Radio Engineers 25, 977-991 (1937).

LEWIS, E. R., Y. Y. ZEEVI and F. S. WERBLIN: Scanning Electron Microscopy of Vertebrate Visual Receptors. Brain Research 15, 559-562 (1969_a).

LEWIS, E. R., T. E. EVERHART and Y. Y. ZEEVI: Studying Neural Organization in Aphysia with the Scanning Electron Microscope. Science 165, 1140-1143 (1969_b).

LEWIS, E. R.: Studying Neuronal Architecture and Organization with the Scanning Electron Microscope. In: Scanning Electron Microscopy, Johari, O., Ed.: 281-288. I.I.T. Research Institute, Chicago, Illinois 1971.

LIM, D. J.: Scanning Electron Microscopic Observation on Non-Mechanically Cryofractured Biological Tissue. In: Scanning Electron Microscopy, Johari, O., Ed.: 257-264. I.I.T. Research Institute, Chicago, Illinois 1971.

LIPKIN, B. L. and A. ROSENFELD: Picture Processing and Psychopictorics, 526 pp. New York: Academic Press Inc., ~~N. Y.~~ 1970.

MAC DONALD, N. C.: Computer-Controlled Scanning Electron Microscopy. In: Proc. 26th Ann. Meeting Electron Microscope Soc. Amer. Arceneaux, C., Ed.: 362. Claitor's Publishing, New Orleans, 1968.

MAC DONALD, N. C.: Auger Electron Spectroscopy for Scanning Electron Microscopy. In: Scanning Electron Microscopy, Johari, O., Ed., 89-96. I.I.T. Research Institute, Chicago, Illinois 1971.

MANGER, W. M. and M. BESSIS: Cathodoluminescence Produced in Cells and Proteins by Paraformaldehyde as Seen with the Scanning Electron Microscope. Septieme Congres International de Microscopie Electronique, Grenoble, 483-484. Societe Francaise de microscopie Elecl., Paris 1970.

MANNHEIM, L. A.: Stereoscopic System for Any Size Picture Pairs (Nesch vertical system), Photographic Applications in Sci., Technol. and Med., 38, Nov. (1971).

MANNING, J. S., T. L. HAYES and R. M. GLAESER: Comparison of Transmission and Scanning Electron Microscope Images of Small Biological Objects. Biophys. J. 8, A159 (1968).

MARSZALEK, D. S. and E. B. SMALL: Preparation of Soft Biological Materials for Scanning Electron Microscopy. In: Scanning Electron Microscopy, Johari, O., Ed., 231-239. I.I.T. Research Institute, Chicago, Illinois 1969.

MATAS, B. R., W. H. SPENCER, T. L. HAYES and R. D. CHANDLER: Morphology of Experimental Vaccinial Superficial Punctate Keratitis -- A Scanning and Transmission Electron Microscope Study. Invest. Ophthal. 10, 348-356 (1971) and Private Communication 1971.

MC ALEAR, J. H., O. KREUTZINGER and R. F. W. PEASE: Combining Scanning and Transmission Electron Microscopy in the Study of Heart Muscle Freeze-Etched Replicas. J. Cell. Biol. 35, 89A (1967).

MC CALLISTER, L. P. and R. HADEK: Transmission Electron Microscopy and Stereo Ultrastructure of the T System in Frog Skeletal Muscle. J. Ultra-Struct. Res. 33, 360-368 (1970).

MC DONALD, L. W., R. F. W. PEASE and T. L. HAYES: Scanning Electron Microscopy of Sectioned Tissue. Lab. Invest. 16, 532-538 (1967).

MC DONALD, L. W. and T. L. HAYES: The Role of Capillaries in the Pathogenesis of Delayed Radionecrosis of Brain. Amer. J. Path. 50, 745-764 (1967).

MC DONALD, L. W. and T. L. HAYES: Correlation of Scanning Electron Microscope and Light Microscope Images of Individual Cells in Human Blood and Blood Clots. Exp. Molec. Path. 10, 186-198 (1969).

MICHAELIS, T. W., N. R. LANIMER, E. N. METZ, and S. P. BALCERZAK: Surface Morphology of Human Lekocytes. Blood, 37, 23-30 (1971).

MUIR, M. D., P. R. GRANT, G. HUBBARD, and J. MUNDELL: Cathodoluminescence Spectra. In: Scanning Electron Microscopy, Johari, O., Ed., 401-408. I.I.T. Research Institute, Chicago, Illinois 1971.

NEMANIC, M.: Private communication, 1971.

NIXON, W. C.: Twenty Years of Scanning Electron Microscopy, 1948-1968, in the Engineering Department, Cambridge University, England. In: Scanning Electron Microscopy, Johari, O., Ed., 55-62. I.I.T. Research Institute, Chicago, Illinois 19[illegible].

NOWELL, J. A., J. PANGBORN, and W. D. TYLER: Scanning Electron Microscopy of Avian Lung. In: Scanning Electron Microscopy, Johari, O., Ed., 249-256. I.I.T. Research Institute, Chicago, Illinois 1970.

OATLEY, C. W., W. C. NIXON and R. F. W. PEASE: Scanning Electron Microscopy. Adv. in Electronics and Electron. Physics, Vol. 21, 181-247 (1965).

PARDUCZ, B.: Ciliary Movement and Coordination in ciliates. Int. Rev. Cytol. 21, 91-128 (1967).

PAWLEY, J. B., F. T. UPHAM, A. A. WINDSOR, and T. L. HAYES: Color Modulation Display and an Exposure Measuring Circuit for Scanning Electron Microscope Images. Univ. of Calif. Radiation Lab. Report, UCRL 19420 (1969) Fall Semi-Annual Report, Berkeley, 68-76.

PAWLEY, J. B. and T. L. HAYES: A Micromanipulator for the Scanning Electron Microscope. In: Scanning Electron Microscopy, Johari, O., Ed.: 105-112. I.I.T. Research Institute, Chicago, Illinois 1971.

PAWLEY, J. B.: Ph.D. Thesis, University of California, Berkeley, in press 1972_a.

PAWLEY, J. B.: A Dual Needle Piezoelectric Micromanipulator for the Scanning Electron Microscope. Rev. Sci. Instr., 43 (4), April 1972_b.

PAWLEY, J. B.: Charging Artifacts in the Scanning Electron Microscope. In: Scanning Electron Microscopy, Johari, O., Ed.: I.I.T. Research Institute, Chicago, Illinois, in press 1972_c.

PEASE, D. C.: Histological Techniques for Electron Microscopy, 1-381. New York: Academic Press, Inc., N. Y. 1964.

PEASE, R. F. W. and W. C. NIXON: High Resolution Scanning Electron Microscopy. J. Sci. Instr. 42, 81-85 (1965).

PEASE, R. F. W. and T. L. HAYES: Some Biological Applications of Scanning Electron Microscopy. 6th Int. Congress for Electron Microscopy, Kyoto, Japan 19-20. Maruzen Co., Lt., Tokyo 1966_a.

PEASE, R. F. W., and T. L. HAYES: Scanning Electron Microscopy of Biological Material. Nature (Lond.) 210, 1049 (1966_b).

PEASE, R. F. W., T. L. HAYES, A. S. CAMP and N. M. AMER: Electron Microscopy of Living Insects. Science 154, 1185-1186 (1966).

PEASE, R. F. W. and T. L. HAYES: Scanning Electron Microscopy Using Cathodoluminescence. In: Proc. 25th Ann. Meeting Electron Microscope Soc. Amer. Arceneaux, C., Ed.: 122-123. Claitor's Publishing, New Orleans: 1967.

PEASE, R. F. W. and T. L. HAYES: Electron Microscopy of Sprouting Seeds. In: Proc. 26th Ann. Meeting Electron Microscope Soc. Amer, Arceneaux, C., Ed.: 88-89. Claitor's Publishing, New Orleans: 1968_a.

PEASE, R. F. W. and T. L. HAYES: Biological Applications of the Scanning Electron Microscope. In: Johari, O., Ed., 143-154. I.I.T. Research Institute, Chicago, Illinois 1968b).

PEASE, R. F. W.: Fundamentals of Scanning Electron Microscopy. In: Scanning Electron Microscopy, Johari, O., Ed., 9-16. I.I.T. Research Institute, Chicago, Illinois 1971.

PFEFFERKORN, G.: Preparation Methods and Artifacts in Scanning Electron Microscopy. Proc. 2nd Ann. Stereoscan Colloquium, 81-88. Engis Equipment Co., Morton Grove, Illinois 1969.

POH, T., R. L. J. ALTENHOFF, S. ABRAHAM and T. L. HAYES: Scanning Electron Microscopy of Myocardial Sections Originally Prepared for Light Microscopy. Exp. Molec. Path. 14, 404-407 (1971).

RASHEVSKY, N.: Topology and Life: In Search of General Mathematical Principles in Biology and Sociology. Bull. Math. Biophys. 16, 317-348 (1954).

RASHEVSKY, N.: Mathematical Principles in Biology, 81-114, Charles C. Thomas, Springfield, Illinois 1961.

REMOND, G., S. KIMOTO and H. OKAZUMI: Use of the SEM in Cathodoluminescence Observations in Natural Samples. In: Scanning Electron Microscopy, Johari, O., Ed., 33-40. I.I.T. Research Institute, Chicago, Illinois 1970.

ROTH, I. L.: Scanning Electron Microscopy of Bacterial Colonies. In: Scanning Electron Microscopy, Johari, O., ed., 321-328. I.I.T. Research Institute, Chicago, Illinois 1971.

RUSS, J. C.: Progress in the Design and Application of Energy Dispersion X-ray Analyzers for the SEM. In: Scanning Electron Microscopy, Johari, O., Ed., 65-72. I.I.T. Research Institute, Chicago, Illinois 1971.

SALSBURY, A. J. and J. A. CLARKE: New Method for Detecting Changes in the Surface Appearance of Human Red Blood Cells. J. Clin. Path. 20, 603-610 (1967).

SANDERS, J. V.: Colour of Precious Opal. Nature 204, 1151-1153 (1964).

SCHEID, W. and H. TRAUT: Visualization by Scanning Electron Microscopy of Achromatic Lesions ("gaps") Induced by X-Rays in Chromosomes of Vicia faba. Mutation Res. 11, 253-255 (1971).

SCHEIE, P. and H. DAHLEN: Surface Features of Cultured Mammalian Cells. In: Scanning Electron Microscopy, Johari, O., Ed., 127-134. I.I.T. Research Institute, Chicago, Ill. 1969.

SIKORSKI, J., J. S. MOSS, P. H. NEWMAN and T. BUCKLEY: A New Preparation Technique for Examination of Polymers in the Scanning Electron Microscope. J. Sci. Instr. 1, 29-31 (1968).

SMALL, E. B. and MARSZALEK: Scanning Electron Microscopy of Fixed, Frozen and Dried Protozoan. Science 163, 1064-1065 (1969).

SMITH, V., J. W. RYAN, D. D. MICHIE, D. S. SMITH: Endothelial Projections as Revealed by Scanning Electron Microscopy. Science 173, 925-927 (1971).

SOKOLOFF, A., T. L. HAYES, R. F. W. PEASE and M. ACKERMAN: Tribolium Castaneum Morphology of "Aureate" Revealed by Scanning Electron Microscopy. Science 157, 443-445 (1967).

SPENCER, W. H., J. ALVARADO and T. L. HAYES: Scanning Electron Microscopy of Human Ocular Tissues: Trabecular Meshwork. Invest. Ophthal. 7, 651-662 (1968).

SPENCER, W. H. and T. L. HAYES: Scanning and Transmission Electron Microscope Observations of the Topographic Anatomy of Dendritic Lesions in the Rabbit Cornea. Invest. Ophthal. 9, 183-195 (1970).

STACKPOLE, C. W., T. AOKI, E. A. BOYSE, L. J. OLD, J. LUMLEY-FRANK and E. DE HARREN: Cell Surface Antigens: Serial Sectioning of Single Cells as an Approach to Topographical Analysis. Science 172, 472-474 (1971).

STEERE, R. L.: Freeze-Etching Simplified. Cryobiology 5, 306-323 (1969a).

STEERE, R. L.: Freeze-Etching and Direct Observation of Freezing Damage. Cryobiology 6, 137-150 (1969b).

STEERE, R. L.: High Resolution Stereography of Compelementary Freeze-Fracture Replicas Reveals the Presence of Depressions Opposite Membrane Associated Particles of Split Membranes. In: 29th Ann. Proc. Electron Microscopy Soc. Amer., Boston, Mass. Arceneaux, C. Ed.; 442-445. Claitor's Publishing, New Orleans 1971a.

STEERE, R. L.: Retention of 3-Dimensional Contours by Replicas of Freeze-Fractured Specimens. In: 29th Ann. Proc. Electron Microscopy Soc. Amer., Boston, Mass. Arceneaux, C., Ed., 242-243. Claitor's Publishing, New Orleans; 1971b.

STEWART, A. D. G. and A. BOYDE: Ion Etching of Dental Tissues in A Scanning Electron Microscope. Nature 196, 81-82 (1962). (Lond.)

SUTFIN, L. V., M. E. HOLTROP and R. E. OGILVIE: Microanalysis of Individual Mitochondrial Granules with Diameters Less than 1,000 Angstroms. Science 174, 947-949 (1971).

SWIFT, J. A. and A. C. BROWN: Transmission Scanning Electron Microscopy of Sectioned Biological Materials. In: Scanning Electron Microscopy, Johari, O. Ed.; 113-120. I.I.T. Research Institute, Chicago, Ill. 1970.

THOMAS, R. S.: Microincineration Techniques for Electron-microscopic Localization of Biological Material. In: Advan. in Opt. and Electron Microscopy, Barer, R. and Cosslett, V. E. Eds.; Vol. 3, 99-150. Academic Press, London; 1969.

THORNHILL, J. W., R. K. MATTA, and W. H. WOOD: Examining Three-Dimensional Microstructures with the Scanning Electron Microscope. Grana Palynologica 6, 3-6 (1965).

THORNTON, P. R.: Scanning Electron Microscopy -- Applications to Materials and Device Science, 34-38, 207-212. Barnes & Noble, Inc. N. Y. 1968.

TOUSIMIS, A. J.: Combined Scanning Electron Microscopy and Electron Probe Microanalysis of Biological Soft Tissue. In: Scanning Electron Microscopy, Johari, O., Ed.; 217-230. I.I.T. Research Institute, Chicago, Illinois 1969.

WELLS, O. C.: Correction of Errors in Electron Stereomicroscopy. Brit. J. Appl. Phys. 11, 199-201, 1960.

WELLS, O. C.: Bibliography in the Scanning Electron Microscope. In: Record of the Inst. Elect. Electron Engnrs. 9th Symposium on Electron, Ion and Laser Beam Technology, Pease, R. F. W., ed., 412-438. I.E.E.E. 1967.

WELLS, O. C.: Bibliography on the Scanning Electron Microscope. In: Scanning Electron Microscopy, Johari, O., Ed., SEM Symposium 1968, 1969, 1970, 1971, I.I.T. Research Institute, Chicago, Illinois.

WITTRY, D. B. and A. VAN COUVERING: A Stereoscopic Display System for Electron Microprobe Instruments. J. Sci. Instr. 44, 294-295 (1967).

ZEITLER, E.: Resolution in Electron Microscopy. In: Adv. in Electronics and Electron Physics, Marton, L., Ed.; 25, 277-332. Academic Press, N. Y. 1969.

ZEITLER, E.: Scanning Transmission Electron Microscopy. In: Scanning Electron Microscopy, Johari, O., Ed.; 25-32. I.I.T. Research Institute, Chicago, Illinois 1971.

WARFEL, A. H. and S. S. ELBERG: Macrophage Membranes Viewed Through a Scanning Electron Microscope. Science, 170, 446-447 (1970)

FIGURE CAPTIONS

Fig. 1. Information coding. The amount of information transferred by an image can depend on qualities other than resolution. Two images of an identical, nearly random, alphabet sequence are shown at the same resolution. The ability to perceive the message in the lower image depends more on qualities such as contrast and tone than on any parameter related to resolution or detail.

Fig. 2. Stereoscopic viewing. By adding the code of binocular depth and shape perception to the shading and overlap codes usually seen with single scanning electron micrographs, an enhancement of subjective contact as well as resolution of analytic ambiguities can be achieved. These two stereo-pairs (X 300) represent two views of a two-hundred mesh woven metal grid used in the preparation of electron microscope samples. There are approximately two hundred wires to the inch. Our understanding of the system is enhanced by the addition of the stereoscopic imaging which is added to an already vivid characterization by means of the shading contrast seen in individual members of the pair of pictures.

In order to aid in the viewing of stereo-pairs presented in this chapter it is suggested that the reader construct a small, inexpensive prism (see Appendix #1). The pair of pictures as viewed through the prism with the right eye will be displaced vertically downward as compared to the image viewed simultaneously by the left eye which looks at the picture directly without any optical device. By changing the distance between the prism and the page, the picture intended for the right eye (R) will merge with that for the left (the "R" will overlap the "L") allowing assimilation of stereoscopic information. A non-stereoscopic image for comparison will be seen above and below the center, superimposed image when the page is viewed in this way.

Fig. 3. Biological organization in depth. Stereo-pairs (X 1100) showing the growth of organisms on a soft contact lens. (Spencer, et al) When viewed stereoscopically, the organism (Aspergillus fumagatus) exists as strands reaching high off the surface of the lens material. Note that strands on the surface (s) can be distinguished from strands (h) high above the surface. Also note nearly vertical post (p) difficult to recognize in single picture.

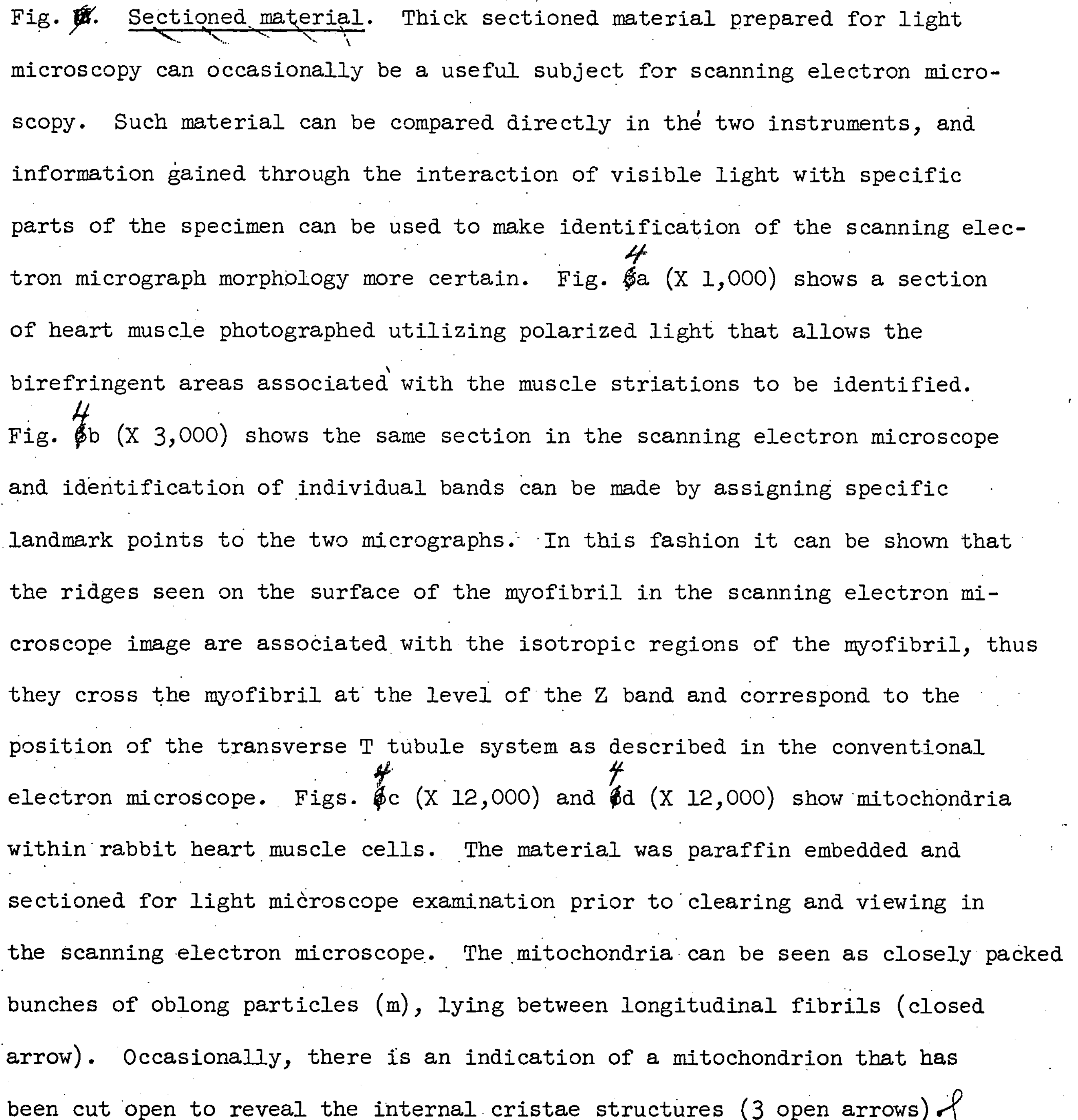

Fig. 4. Sectioned material. Thick sectioned material prepared for light microscopy can occasionally be a useful subject for scanning electron microscopy. Such material can be compared directly in the two instruments, and information gained through the interaction of visible light with specific parts of the specimen can be used to make identification of the scanning electron micrograph morphology more certain. Fig. 4a (X 1,000) shows a section of heart muscle photographed utilizing polarized light that allows the birefringent areas associated with the muscle striations to be identified. Fig. 4b (X 3,000) shows the same section in the scanning electron microscope and identification of individual bands can be made by assigning specific landmark points to the two micrographs. In this fashion it can be shown that the ridges seen on the surface of the myofibril in the scanning electron microscope image are associated with the isotropic regions of the myofibril, thus they cross the myofibril at the level of the Z band and correspond to the position of the transverse T tubule system as described in the conventional electron microscope. Figs. 4c (X 12,000) and 4d (X 12,000) show mitochondria within rabbit heart muscle cells. The material was paraffin embedded and sectioned for light microscope examination prior to clearing and viewing in the scanning electron microscope. The mitochondria can be seen as closely packed bunches of oblong particles (m), lying between longitudinal fibrils (closed arrow). Occasionally, there is an indication of a mitochondrion that has been cut open to reveal the internal cristae structures (3 open arrows).

Fig. 5. Standard specimens and drying artifacts. Precious opal can be utilized as a convenient standard for the assessment of resolution and stability of the scanning electron microscope. In addition to the array of spherical units visible at a moderate degree of magnification, (Fig. 5a, X 19,000) a very fine internal structure associated with each individual sphere can be seen at higher magnifications (Fig. 4b, X 55,000). The spherical particles also offer a very useful geometry for the correction of astigmatism (see also Fig. 8).

The effects of air drying as compared to freeze drying can be seen in the lower pair of photographs (X 21,000) showing the corneal epithelium of the rabbit (MATAS, et al., 1971). In Fig. 5c the microvilli are quite well preserved following freeze-drying, while in Fig. 5d the surface has been greatly flattened and detail has been lost following the air drying process.

Fig. 6. Charging artifact. (J. Pawley) Fig. 6ab (X 100) is a stereo-pair showing the micro-stub used for holding specimens at a fixed potential. Again, a prism can be used to superimpose "R" (right eye) with "L" (left eye) for stereo viewing. The small stub is electrically isolated and can be set at a potential different from that of the surrounding surface. In this way, many of the charging artifacts can be produced under controlled conditions. Fig. 6c (X 300) shows a small fragment of the wing of the flour beetle (Tribolium confusum) attached to the micro-stub and Fig. 6d (X 10,000) illustrates the setae (bristles) on this wing as the specimen is held at a potential of minus 90 volts. Binocular addition of grey levels can also be illustrated by simultaneously stereo viewing the bright (6b) and dark (6a) band at the top of the pair.

Fig. 7. Specimen current and resolution. Specimen current is an important parameter determining, in part, the available resolution of the SEM image. Figs. 7a (X 10,000) and 7b (X 10,000) compare the resolution at specimen currents of 1 x 10^{-10} amps and 0.7 x 10^{-11} amps respectively. For maximum resolution, the specimen current must be reduced to the lowest value consistent with a relatively noise free image.

In order to determine some of the quantitative results of variations in specimen current, beam current, and collected secondary electron current, it can sometimes be useful to utilize a small stage mounted electromagnet (J. Pawley) to deflect the beam directly onto the scintillator. Characteristics of the raster are maintained quite well as shown by the imaging possibilities of such a deflected beam. In Fig. 7c the scintillator (s) and surrounding collector shield (c) can be seen at a magnification of less than one, (X 0.5). In Fig. 7d (X 1) a detail of the stage mount can be recognized after deflection of the beam by the stage mounted magnet. Calibration of the scintillator-photomultiplier can be carried out by such direct deflection of the incident beam to the collector after first measuring the beam current with a Faraday cup specimen.

Fig. 8. Astigmatism correction. Astigmatism in the scanning electron microscope image is a common limitation to resolution. The operator can correct this aberration by applying an astigmatism that is equal but opposite to the inherent astigmatism found in the instrument at that time. A rectilinear grid inscribed on a transparent plastic disk is placed over the face of the display cathode ray tube and is rotated in order to locate and mark the direction of astigmatism during the correction procedure. Astigmatism means that different directions focus at different points. We can see in Figs. 8a and 8b that the images are smeared in one direction but sharp at 90 degrees to that direction and that the directions reverse between Fig. 8a (low focus) and 8b (high focus). If we attempt to take an intermediate focus position, we find that there is no position which will give good resolution. Fig. 8c represents an attempt at a best focus, non-corrected image. Fig. 8d shows the beneficial effect of astigmatism correction. Now all directions focus at the same point and the resolution of the micrograph is considerably improved (Fig. 8a, b, c, d, opal, all X 33,000).

(J. Pawley)

Fig. 9. Micromanipulation. Fig. 9 demonstrates micromanipulation within the column of the scanning electron microscope. Fig. 9a (X 40) shows two independently controlled needles (n1, n2) in contact with the surface of the small insect Tribolium confusum. The needles can operate as fine microelectrodes for the controlled application of electrical potential and can be quite useful for localized grounding of nonconducting specimens. Fig. 9b (X 2,000) shows the more normal use of one of the dissection needles (n1) to separate tissue while it is being viewed in the scanning electron microscope. This picture was made by photographing the face of a TV scan rate display tube. The lower photographs (Fig. 9c X 10,000, 9d X 10,000) illustrate two complementary surfaces that were obtained by separating layers of tissue from the stomach of the frog. Such complementary surfaces are somewhat analogous to those achieved through certain freeze-etching techniques. In Fig. 9d (right), the small villi of the surface are represented as holes in the facing surface.

Fig. 10. Geometric transformations. Projective or topologic transformations must be assessed with some care in the interpretation of the images seen in the scanning electron microscope. Figs. 10a (X 3,700) and 10b (X 3,700) are "uncorrected" images of spherical cells taken at 45 degree specimen tilt. Figs. 10c and 10d are the corresponding "corrected" images after applying commercially available electronic modification of the raster to take into account the foreshortening of planar measurements taken at 45° tilt.

Since the aspect presented in any scanning electron microscope image is that of observation from the electron gun, (line of sight parallel to the probing beam), it is clear that the measurements on a tilted plane, as projected from this viewpoint are, in fact, a simple function of the angle of the tilt. However, three dimensional objects such as a spherical cell, although resting on the tilting plane, may not be projected according to this same function. A sphere, for example, remains circular in projection regardless of the angle of tilt. Thus, in these pairs of images the circular uncorrected image is accurate whereas the electronic tilt correction has introduced serious distortion into the image. The fact that the long axis of all particles are in the direction of the tilt and that the axial ratio can be calculated from the angle of tilt (on the assumption of spheres as true shapes) gives a strong indication that the "corrected" images are products of an artificial distortion. In any application of geometric factors, it is necessary that the proper dimensional qualities are considered if we are to avoid spurious descriptions (1 μ mark shown.)